THE UNIVERSITY OF MANITOBA

A CORRELATION BETWEEN DEFECTIVE GLYCOPROTEIN BIOSYNTHESIS, ALTERED CELL SURFACE GLYCOPROTEINS AND A NONMYOGENIC PHENOTYPE IN CONCANAVALIN A-RESISTANT L6 MYOBLASTS

By

CRAIG PARFETT

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements for the Degree

Doctor of Philosophy

Department of Microbiology

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To Mary, my Mother and Father.

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SOURCES OF MATERIAL

Most biochemicals and enzymes were purchased from Sigma Chemical Co., St. Louis and most radiochemicals from New England Nuclear, Boston.

Cell Growth Material	Source
α-minimal essential medium	Flow Labs
foetal bovine, calf and horse serum	GIBCO
plastic culture plates	Lux Scientific Corporation
glass culture plates	Fisher Scientific Company
Bactotrypsin	Difco

Experimental Material	Source
sodium dodecyl sulfate	British Drug House
concanavalin A	Calbiochem
diadenosine pentaphosphate	Boerhinger Manheim
25 hydroxycholesterol	Steraloids
phenol	J.T. Baker Chemical Company
2,5 diphenyloxazole (PPO)	Fisher Scientific Company
1,4-bis(2-(5-Phenyloxazolyl)) Benzene (POPOP)	Fisher Scientific Company
Phenethyl Alcohol	Matheson, Coleman & Bell
Tween 80	Matheson, Coleman & Bell
Zinc chloride	Matheson, Coleman & Bell
Dextran T-500	Pharmacia

NCS Tissue Solubilizer	Amersham
Triton X-100	J.T. Baker Chemical Co.
2-Mercaptoethanol	Eastman
N,N,N,N-tetramethyl ethylene diamine	Eastman
ammonium persulfate	J.T. Baker Chemical Company
GDP-[^{14}C] mannose	Amersham

LIST OF ABBREVIATIONS

1	Asn	asparagine
2	con A	concanavalin A
3	EDTA	ethylene diamine tetraacetic acid
4	Fuc	fucose
5	GalNAc	N-acetylgalactosamine
6	GDP	guanosine diphosphate
7	Glc or G	glucose
8	GlcNAc	N-acetylglucosamine
9	HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
10	LCA	*Lens culinaris* agglutinin
11	Man or M	mannose
12	αMEM	α minimal essential medium
13	PHA	phytohemagglutinin
14	PBS	phosphate buffered saline
15	RPE	relative plating efficiency
16	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
17	Ser	serine
18	TBS	Tris buffered saline
19	Thr	threonine
20	Tris	Tris (hydroxymethyl) amino methane
21	UDP	uridine diphosphate
22	WGA	wheat germ agglutinin

ABSTRACT

Four independent rat L6 myoblast cell lines have been selected in a single step for resistance to the cytotoxic effects of the lectin concanavalin A. In contrast to parental wild type myoblast lines, all of the variant clones are unable to undergo normal cellular differentiation to form multinucleated myotubes or biochemical differentiation to produce an increase in the specific activity of the muscle-specific enzyme, creatine phosphokinase. The correlation between lectin resistance and loss of fusion potential is very tight; clonal variation studies show that there is less than a 2.8×10^{-8} chance that the two are not directly related. Membrane preparations from the concanavalin A-resistant myoblast lines incorporate significantly less GDP-[^{14}C] mannose. Incorporation into exogenous lipid by membranes from variant and wild type myoblasts indicated that the biosynthetic lesion likely involved a mannosyl transferase enzyme directly, rather than a lack of free dolichol-phosphate. Also, conversion of mannose label to fucose occurs in myoblasts and this pathway is more active in concanavalin A-resistant cells than wild type cells. Reduced binding of labelled concanavalin A to the cell surfaces of variant myoblasts was observed which may result from alterations to membrane glycoprotein receptors.

[^{3}H]con A binding studies indicated that the variant myoblasts bound significantly less lectin than wild type cells at 4^{o}C and at 37^{o}C. Scatchard analysis revealed two general types of binding sites (high

and low affinity sites) on wild type cells; the variants appeared to be deficient in the high affinity sites. These changes in con A binding are likely to play an important role in determining the relative resistance of the variant lines to con A cytotoxicity. Lectin binding results could be significantly modified by altering the composition of the serum in the growth medium used to culture myoblasts prior to performing binding experiments, suggesting the existence of productive and nonproductive lectin binding sites on the cell surface. SDS slab gel electrophoresis of [^{3}H] mannose labelled surface membranes prepared from variant and wild type cells showed that several glycoproteins of the con A-resistant myoblasts were defective in mannosylation.

Although direct "staining" of membrane glycoproteins on SDS gels after electrophoresis, did not reveal significant changes in the pattern of bands on wild type and variant cells, the con A binding ability of a pronase digest of one of the altered regions of [^{3}H] mannose incorporation from variant separations, with a molecular weight of 44,500 d, was found to contain glycopeptides with reduced affinity for the lectin, supporting the idea that variant membranes are deficient in a set of high affinity lectin binding sites. The studies link con A resistance, cell surface glycoprotein alterations, and defective mannosyl transferase activity with the inability to carry out normal cellular differentiation to form multinucleated myotubes. Lectin-resistant myoblasts should be useful systems for investigating what appears to be a pleiotropic mutation affecting the myogenesis

process through membrane modifications.

Also, studies were performed to determine whether fibroblasts from patients with Duchenne muscular dystrophy, a disease hypothesized to result from a systemic membrane defect, bind [^{125}I] con A to their surfaces in a different manner than normal, age-matched fibroblasts. No differences could reproducibly be detected among the various pairs examined.

INTRODUCTION

INTRODUCTION

The animal cell cytoplasmic membrane functions as the interface between the precisely regulated internal physical and chemical composition, and the potentially fluctuating external environment. Beyond the ability to sense the state of the external medium, it is the membrane structure which controls the extent to which internal and external environments interact. A complex repertoire of intercellular interactions has been superimposed on these basic functions in multicellular animals. It is well known that the tissue cell plasma membrane plays a key regulatory role in cellular events such as the immune response, intercellular communication, cellular differentiation and in establishing the neoplastic condition.

Much more information about relationships between membrane structures and functions is required before the complex regulatory mechanisms associated with the surface membrane are properly understood. One approach to unlocking the connections between membrane structure and function has been to select and biochemically characterize mutant cells which are defective in either membrane structures or functions. The appeal that this approach presents is that the multiplicity and specificity of changes that can result from mutational events far exceeds those that can be induced by physical or chemical agents. Somatic cell mutant selection is now a fundamental technique applied in cell biology studies, and has resulted in the accumulation of a variety of cells displaying altered, but stable, phenotypes in culture.

These include temperature sensitive, auxotrophic, drug resistant and lectin resistant phenotypes as the most frequently described examples.

This laboratory has been involved, over the last ten years, with developing selection procedures for mammalian cell mutants cultured *in vitro* and with the genetic and biochemical characterization of those cells (Wright, 1979; Wright *et al.*, 1980; Wright *et al.*, 1981). The selection of cell lines resistant to the cytotoxic lectin concanavalin A provided an important opportunity to study cytoplasmic membrane mutants which displayed a complex phenotype characterized by alterations in membrane associated properties including altered cellular morphology and adhesiveness, increased sensitivity to membrane active agents, altered lectin-receptor mobility and cellular agglutination properties, differences in binding mechanism, a decrease in total lectin bound, and altered surface glycoproteins.

The cell line employed in these selections was the Chinese hamster ovary line, a transformed cell capable of infinite growth in culture but demonstrating few interactions in culture that directly represent those that characterize the specialized somatic cells in tissues. During the period in which this work was done however, several permanent cell lines, characterized by the ability to undergo spontaneous differentiation in culture, were isolated by investigators in other laboratories. The adipogenic 3T3 line, the Friend erythroleukaemic line and several myogenic lines are among the most well-characterized. The latter cell lines develop from mononucleated myoblasts into multinucleated syncyctia displaying many structural and physiological attributes of muscle tissue. The myoblast plasma membrane

undoubtedly plays an important regulatory role during muscle development, including the striking morphological transformation of membrane fusion and probably also during initiation of the differentiation program as the myoblasts grow to confluence.

This study was initiated to employ the myogenic cell lines in a selection for variants with altered cell surface properties as a means with which to probe the function of structures at the myoblast cell surface in the differentiation process. Con A was chosen as the selective agent because encouraging results gained from the previous work with the hamster cell lines suggested that participation of surface membrane glycoproteins is important for normal behaviour of cells during events in which the plasmalemma is thought to play a central role. Several independent selections for myoblasts capable of growing in cytotoxic concentrations of con A were made from wild-type myogenic populations. At a low frequency, con A-resistant cells were isolated and observed to display a stable resistance to the lectin. Interestingly, the variant myoblasts were consistently unable to undergo morphological or biochemical differentiation to form muscle tissue. A comparison of the wild type populations with their respective variants revealed several other less striking alterations in cellular biology. Nevertheless, significant surface alterations were detected including lectin binding characteristics and glycoprotein composition. To account for the pleiotropic nature of the altered phenotype, it was necessary that the biochemical basis for the cell surface defect be understood. The protein glycosylation pathway involving lipid oligosaccharide intermediates was examined and observed to transfer mannose

to proteins with reduced efficiency. It is likely that the absence of a single mannosyl transferase activity was responsible for the cellular phenotype displayed in the variants that were selected. These results are discussed with reference to the possible structural components on the myoblast cell surface that may play a role during the developmental process.

HISTORY

HISTORY

A Role For Glycoproteins in Cell Surface Membrane Functions

The plasma membrane of eucaryotic cells is currently conceived to consist of a fluid, amphipathic lipid bilayer into which is integrated by both hydrophobic and electrostatic forces, a diverse assemblage of protein molecules. The formal presentation of the fluid mosaic membrane model by Singer and Nicholson (1972) stressed the concept of a role for the protein components beyond merely structural. Evidence was presented to substantiate the claim that polypeptides embedded within the lipid bilayer were free to interact not only in the plane of the membrane, but also with the molecular environment encountered either externally or internally, depending on the orientation of the specific protein under consideration. Further, an important prediction was made of a class of protein molecules spanning the membrane and playing central roles in the interactions of a cell with its environment, by virtue of these molecules' potential to mediate transmembrane signals.

The dynamic nature of membrane composition and configuration provides a conceptual framework from which to fashion the testable hypotheses needed to probe the intricate and delicate control mechanisms involved during cellular responses to external stimuli. The eucaryotic cytoplasmic membrane has many known functions of this nature and there are others that are either wholly unknown or only

suspected. Such functions include: active and facilitated transport of specific ions and molecules, cellular recognition and adhesion, the immune response, regulation of cell growth, cellular differentiation, a receptor for viruses or for hormones and other substances with pharmacological effects, engulfment of particles from the environment, and establishment of the neoplastic condition.

Considerable evidence implicates the participation of the membrane glycoproteins in all of these important biological functions. The experimental evidence has been derived in basically three ways: a) by demonstration that a protein which has been observed to be a component of a particular function is also covalently linked to an oligosaccharide chain, b) by observing regulatory shifts in membrane glycoprotein composition during the time in which the membrane activity occurs, and c) by studying the consequences of chemical modification or metabolic and genetic blocks of glycosylation. Thus, cell surface glycoproteins have been shown to play important roles in pinocytosis (Neufeld and Ashwell, 1980), differentiation (Suyama and Goldstein, 1982; Senechal *et al.*, 1982; Moscona, 1974; Barondes, 1980; Parfett *et al.*, 1981), tumorigenesis (Hughes, 1976a; Yogeeswaran, 1980; Robbins and Nicolson, 1975), intercellular recognition and adhesion (Hughes 1976b), intercellular communication (Smets *et al.*, 1982), as receptors for hormones and viruses (Hughes 1976c) and as mediators of immunological specificity (Hughes, 1976d; Hart, 1982).

The evidence for participation of glycoproteins in a variety of important cellular membrane processes has been gathered from a phylogenetically diverse group of organisms. The wide distribution and

conserved structure of the carbohydrate chains suggests their importance in a universal but as yet undefined metabolic process. Several suggestions have been made for a general function provided by covalently-linked oligosaccharide chains, such as labelling of proteins for intracellular recognition and export (Eylar, 1965; Melchers, 1973), recognition of an extracellular secretion product by its target cell (Winterburn and Phelps, 1972) or protection against proteolytic degradation (Gottschalk and Fazekas de St. Goth, 1960). Further experimentation must be done before a decision can be made concerning the general validity of these models. Their chief significance may have been to direct speculation toward a general hypothesis in which the oligosaccharide component provides an informational or recognitional marker to the catalytic or structural functions of the polypeptide to which it is attached. For example, the surface membrane contains receptors for soluble glycoprotein ligands, many of which function as hormones (Hughes, 1976c). Ashwell, Morrell and their coworkers initially described (Morrell *et al.*, 1968), purified and characterized (Hudgin *et al.*, 1974; Kawasaki and Ashwell, 1976a,b), an hepatocyte cell surface receptor (Pricer and Ashwell, 1971) which recognizes desialylated glycoproteins bearing terminal non-reducing galactose residues. This receptor-mediated endocytotic mechanism is responsible for their rapid serum clearance and lysosomal catabolism (Ashwell and Morrell, 1974). It was the first mammalian protein identified as a lectin-like molecule and has since been studied extensively in its binding characteristics (Weigel, 1980) and its ability to be spared lysosomal destruction yet remain available for reinsertion into the plasma

membrane (Steer and Ashwell, 1980).

The phosphomannosyl, receptor-mediated uptake of lysosomal hydrolases at the surface of fibroblasts represents another excellent example of the cognitive role played by oligosaccharides of glycoproteins (for a review see Sly and Fisher, 1982). Neufeld and coworkers in the late 1960s reported that normal fibroblasts secrete "corrective-factors" which overcame the deficiencies of mucopolysaccharidosis (Fratantoni *et al.*, 1969). Later identified as acid hydrolases (Bach *et al.*, 1972), a selective and saturable uptake system was observed to be involved in their ability to overcome the defective oligosaccharide breakdown responsible for these storage diseases (Neufeld *et al.*, 1975). Kaplan *et al.*, (1977) provided the first evidence that lysozomal hydrolases rapidly taken up by cells are phosphoglycoproteins. It was suggested that mannose-6-phosphate was the moiety which confers specificity because treatment of β-glucuronidase with alkaline phosphatase abolished its "high-uptake" activity, and mannose-6-phosphate was a potent competitive inhibitor of that uptake. Direct demonstration that mannose-6-phosphate residues were located on high-mannose oligosaccharides was obtained by von Figura and Klein (1979) and by Natowicz *et al.*, (1979). Removal of high mannose oligosaccharides from the hydrolases by endoglycosidase H, converted the enzyme to a form no longer susceptable to adsorptive pinocytosis.

Although the receptor will bind monophosphorylated mannose derivatives, (Kaplan *et al.*, 1977), high affinity uptake is only achieved with substrates that have many exposed 6-phosphomannose groups

(Fisher *et al.*, 1980a) and offer a possible multivalent interaction with the receptors of the fibroblast surface. Low density lipoprotein, when chemically modified by covalently attaching pentamannosyl phosphate groups acts as an efficient, yet artificial, ligand for the receptor (Murray and Neville, 1980). High mannose groups are normally phosphorylated *in vivo* by the action of two enzymes: a UDP-N-acetylglucosamine: glycoprotein-N-acetylglucosamine-1-phosphate transferase (Hailkik *et al.*, 1981; Reitman and Kornfeld, 1981); and an enzyme that hydrolyses the phosphodiester bond, removing the blocking N-acetyl glucosamine residue (Waheed *et al.*, 1981a,b).

The phosphomannosyl receptor has been purified and characterized (Sahagian *et al.*, 1980, 1981). The fact that only about 20% of the receptor is located on the surface membrane (Fisher *et al.*, 1980b) has led to the suggestion that the major function of the receptors in cells is to regulate the intracellular transport of newly synthesized acid hydrolases (Sly and Fisher, 1982). Nevertheless, this system clearly illustrates how cells make use of an oligosaccharide structure as a specific recognition marker at a membrane surface, in this case for transfer of enzymes into the lysosome.

This idea is particularly well developed among workers investigating the mechanisms of cellular recognition and adhesion. Early indications that specific surface recognition systems did exist began with the observation by Wilson (1907) of species-specific reaggregation of dissociated cells using sponge species mixtures of different colours. An aggregation factor was shown by Moscona (1963) to be released from the sponge cell surface which specifically stimulated the reaggregation

of dissociated cells of the same species. The glycoprotein nature of the aggregation factor became apparent (Moscona, 1963; Humphreys, 1965) with about 50% of the structure being made up of glucosamine, fucose, mannose, galactose and glucose (Margoliash *et al.*, 1965). The oligosaccharide structures were implicated in binding specificity since modification by periodate or digestion with glycosidases destroyed the activity of the factor (Burger *et al.*, 1971; Turner and Burger, 1973). A second macromolecular component, which probably acts as a receptor in the process has also been described (Weinbaum and Burger, 1973).

Extensive characterization of the cellular aggregation properties of the slime mould *Dictyostelium discoideum* has revealed the importance of surface glycoproteins during aggregation and assembly of a multicellular structure (pseudoplasmodium) from individual amoebae (Barondes and Rosen, 1976). A soluble carbohydrate binding protein capable of agglutinating erythrocytes was originally identified in extracts of the aggregating amoebae by Barondes and his coworkers (Rosen, 1972). The protein is present in differentiated, aggregation competent *D. discoideum* cells but is absent in vegetative, non-cohesive amoebae (Rosen *et al.*, 1973; Ma and Firtel, 1978). Several lines of evidence indicate that the protein, named discoidin, is present on the surface of differentiated cells (Chang *et al.*, 1975; Siu *et al.*, 1976). High affinity interaction of discoidin with developmentally regulated oligosaccharide-containing membrane protein receptors may mediate species specific cell adhesion (Reitherman *et al.*, 1975; Bartles and Frazier, 1980). A possible structural mutant of the discoidin gene leads to defective aggregation

of the amoebae, further supporting this hypothesis (Ray *et al.*, 1979).

Roseman (1970) first suggested that cell surface glycosyl transferases may play a role in mediating cellular adhesion. The basis of this model was that glycosyl transferases located on the surface of one cell interact with the glycoprotein carbohydrate moieties or glycolipid on another cell to form a complex with a finite half-life. An example of such a system may exist in the mechanism by which blood platelets aggregate at a breech in blood vessel endothelium. Jamieson *et al.*, (1971) proposed the formation of an adhesive complex between the carbohydrate units of the connective tissue collagen exposed at the injury site and a specific collagen - α glucosyl transferase located on the platelet cell surface. Galactose oxidase treatment of soluble collagen, or periodate oxidation of an active glycopeptide fragment of collagen destroys the aggregative activity, consistent with the role of terminal galactose residues as sites for binding glucosyl transferase (Vermylen *et al.*, 1973; Kang *et al.*, 1974).

Many more examples of the importance of glycoproteins in specific recognition and adhesion have appeared in the literature. For example: influenza virus binding to its host cell requires cell surface sialic acid (Schultz, 1973; Paulson *et al.*, 1979); interaction of the acrosomal process of sea urchin spermatozoa with the egg cell surface (Glabe and Vacquier, 1978; Kinsey and Lennarz, 1981); homing of lymphocytes to various organs (Gesner and Ginsburg, 1964; Gesner, 1966); sexual mating in yeasts (Hughes and Sharon, 1978) and algae (Wiese and Hayward, 1972), and gastrulation during the development of sea urchin embryos (Heifetz and Lennarz, 1979; Carson and Lennarz, 1980). The important work yet

to be done in these studies will be to first identify the components which are involved and to clarify the mechanism by which specificity is achieved.

Other cell membrane biological phenomena are now known to involve cell surface membrane glycoproteins, although the mechanism(s) in which they participate tend to be much less clear than for the examples of recognition and adhesion described above. Cellular growth control has been one of the most intensively studied areas with regard to cell surface carbohydrate. The growth of normal cells in culture is sensitive to the contacts between cells that result when a population begins to crowd the substratum to which it is attached. Weiss and Subjeck (1974) and Gilter *et al.*, (1973) observed sialic acid residue clearance from the points of contact between cells, but it is merely speculation that this event can be considered to be a component of the regulatory response which converts the physical surroundings of the cell to a cytoplasmic signal controlling cellular proliferation. Glycoprotein synthesis must be regulated during the cell cycle, since between mitotic divisions, cells grow to about twice their original size before the next mitosis occurs; therefore, the cellular glycoprotein content must also be increased two-fold. By following the incorporation rates of appropriate biosynthetic precursor molecules into glycoproteins, several workers have observed that glycoproteins are synthesized throughout the cell cycle but that late S and G2 periods are the times of maximum glycoprotein synthesis (Nowakowski *et al.*, 1972; Bosman and Winston, 1970). Glick *et al.*, (1971) have shown that the total cellular content of mannose, fucose and galactose are at a minimum just before

and during mitosis. In contrast, the total cellular and surface membrane sialic acid is at a maximum just before cell division (Glick *et al.*, 1971; Graham *et al.*, 1973; Mayhew, 1967), implying that there are marked differences in the glycoprotein carbohydrate structure throughout the cell cycle.

Warren and his colleagues have identified a high molecular weight, cell surface glycopeptide fraction containing sialic acid that appears only on actively growing cells and in virally transformed cells (Warren *et al.*, 1972; Warren and Buck, 1980). Moreover, the desialylated glycopeptides functioned as acceptors in a trans-sialylation reaction which was seen to be three-fold more active in the transformed cells when compared with their untransformed counterparts. Desialylated fetuin would not serve as a substrate in this reaction, indicating that the presence of a specific transferase of unique function may be activated during transformation. It has not yet been determined if these sialic acid containing structures or transferase activities are regulated or have a specific function during the normal cell cycle. Yogeeswaran (1980) has alternatively suggested that the tumor cell surface sialic acid suppresses the immunogenicity of neoplastic cells by masking their antigenic determinants, thus preventing recognition by the immune system.

The Structure and Biosynthesis of Membrane Glycoproteins

Research into glycoprotein structure and biosynthesis is currently proceding with great activity among many investigators. A brief overview will be presented here with emphasis on oligosaccharide structures that have been described in detail and are normal, animal cell surface membrane components. More comprehensive literature reviews concerning various aspects of structure (Kornfeld and Kornfeld, 1980; Montreuil, 1980; Wagh and Bahl, 1981) and biosynthesis (Parodi and Leloir, 1979; Staneloni and Leloir, 1979; Hubbard and Ivatt, 1981; Staneloni and Leloir, 1982) have recently been published.

Covalently attached oligosaccharide chains is the feature which distinguishes glycoproteins from other polypeptides and accounts for some of their characteristic physical, chemical and biological properties. The carbohydrate content can range from 80% to less than 1% of the molecule. Three sugar types are commonly found: 1) neutral sugars such as D-galactose, D-mannose, D-glucose and L-fucose, 2) the amino sugars N-acetylglucosamine and N-acetylgalactosamine, and 3) acidic sugars or sialic acids. Two linkages have been described between the sugar and polypeptide backbone of the membrane glycoproteins. The N-glycosidic bond, as it has come to be known consists of a glycosylamine bond between the anomeric carbon of the amino sugar N-acetylglucosamine and the amide group of the amino acid asparagine (Fig. 1a,i). The N-glycosidic bond is the most commonly occurring bond among the glycoproteins studied to date and was initially established in ovalbumin by the isolation of the N-acetylglucosaminyl asparagine complex (Marks

et al., 1963; Marshall and Neuberger, 1964). Several membrane glycoproteins containing a second linkage have been described in which the carbohydrate (often N-acetylgalactosamine) is linked through the hydroxyl group of serine, threonine, hydroxylysine or hydroxyproline (Fig. 1b).

These bonds are easily distinguished from the N-glycoside type by susceptibility of the former to cleavage under alkaline conditions by a process known as β-elimination. Many oligosaccharide structures linked in this fashion have been published; for examples see Fig. 1a, ii and iii. Some glycoproteins from erythocyte membranes have been shown to be of the O-glycosidic variety (Tomita and Maachesi, 1975; Thomas and Winzler, 1969), as well as from mouse ascites cells (Jeanloz and Codington, 1974) and melanoma cells (Bhavanandan *et al.*, 1977).

The oligosaccharides of the N-glycosidically linked structures have been divided into two groups based on the fine structure of the chain: a) the high mannose type containing only mannose and N-acetylglucosamine; b) the complex type containing mannose and N-acetylglucosamine as well as galactose, sialic acid, and frequently fucose. The inner core has been found to be the common feature among all asparagine-linked carbohydrates. Thus the designation of the type of carbohydrate structure is defined by the peripheral sugars attached to the inner core. The core structure is as follows:

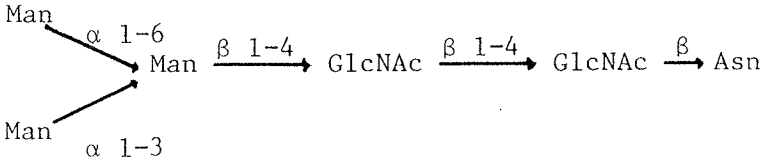


Figure 1a): Structures of the major types of protein-carbohydrate linkages found in glycoproteins.

i) glycosylamine

ii) O-glycoside to serine (or threonine)

iii) O-glycoside to hydroxylysine.

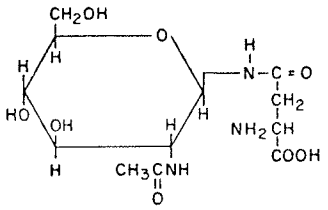
Figure 1b): Structures of O-glycosidically linked oligosaccharides from membrane glycoproteins.

i) epiglycanin (Codington *et al.*, 1975)

ii) blood group substances (Feizi *et al.*, 1971)

a

i)



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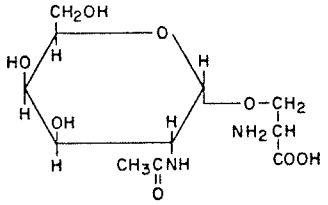
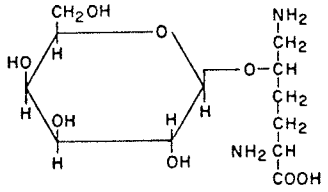
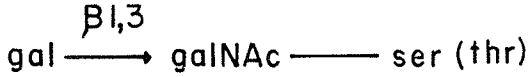


iii)

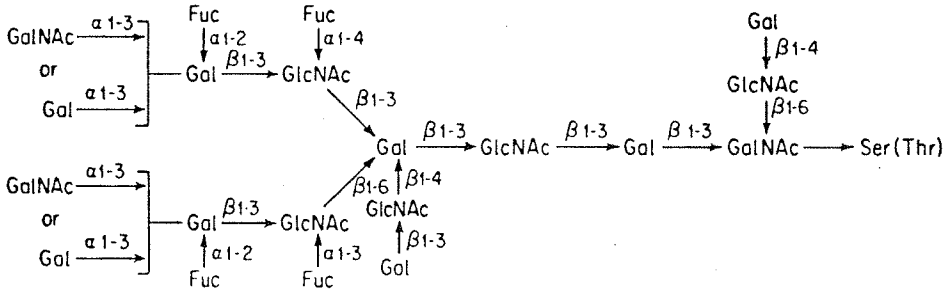


b

i)



ii)



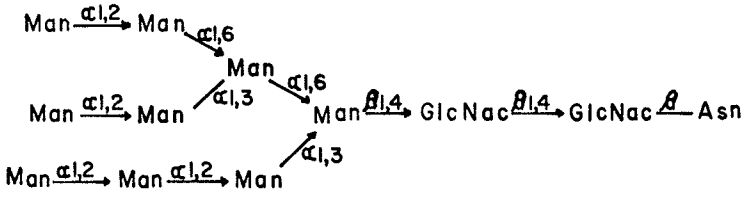
The sequence GlcNAc $\xrightarrow{\beta\ 1\text{-}4}$ GlcNAc$\xrightarrow{\beta}$ Asn was first described by Tarentino *et al.*, (1970) and the mannose residue linked by a β 1-4 glycosidic bond was described originally by Sukeno *et al.*, (1971, 1972).

High mannose oligosaccharides prepared from Chinese hamster ovary cell membrane glycoproteins give a glycopeptide series which varies in composition from $(Man)_5$ $(GlcNac)_2$-Asn to $(Man)_9$ $(GlcNAc)_2$-Asn (Li and Kornfeld, 1979). The same branching pattern of the outer chain α mannosyl residues occurs in all of these structures, including the oligosaccharide containing nine mannose residues which is depicted below (Fig. 2a). Other cell surface glycoproteins that have been determined to contain these oligosaccharide chains are: lymphoma cell Thy 1 antigen (Chapman *et al.*, 1979), and the hepatic membrane-binding protein (Kawasaki and Ashwell, 1976). Oligomannosyl glycopeptides on the cell surface are a major asparagine-linked glycopeptides class in human diploid fibroblasts (Muramatsu *et al.*, 1976; Ceccarini, 1975) and are present in the membrane glycoproteins of SV40-transformed rat fibroblasts (Muramatsu *et al.*, 1975). Neutral glycopeptides containing only mannose and N-acetylglucosamine have also been observed in rabbit fat cell membranes (Kawai and Spiro, 1977), and in Sindbis virus E1 and E2 glycoproteins (Burke and Keegstra, 1979).

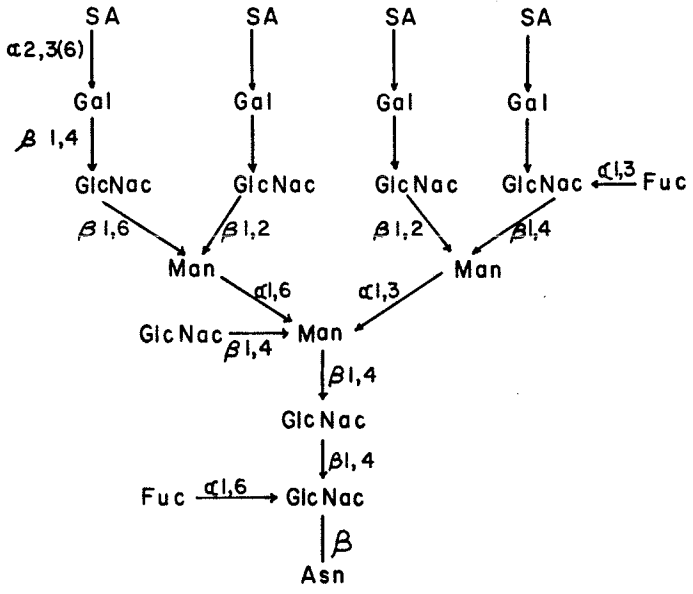
A general structure for the complex type carbohydrate units based on accumulated evidence from carbohydrate structures in various glycoproteins is illustrated in Fig. 2b (Wagh and Bahl, 1981). The structure for almost any carbohydrate unit of this type can be derived from this complete structure by appropriately deleting sialic acid or fucose residues. One or two oligosaccharide chains are attached to each outer

Figure 2: Structures of typical high mannose oligosaccharide (a), and of a complex oligosaccharide (b), linked by a N-glycosidic bond to glycoprotein.

a


Man α1,2 Man α1,6
Man
Man α1,2 Man α1,3
α1,6
Man β1,4 GlcNac β1,4 GlcNac β Asn
Man α1,2 Man α1,2 Man α1,3

b


SA SA SA SA
α2,3(6)
Gal Gal Gal Gal
β 1,4
GlcNac GlcNac GlcNac GlcNac α1,3 Fuc
β 1,6 β 1,2 β 1,2 β1,4
Man Man
α1,6 α1,3
GlcNac β 1,4 Man
β1,4
GlcNac
β1,4
Fuc α1,6 GlcNac
β
Asn

mannose residue of the core structure. Thus the number of such chains present in the carbohydrate structure may be two, three or four, and are commonly refered to as bi-, tri-, or tetra-antenary respectively (Fournet *et al.*, 1978). Complex oligosaccharides have been reported from hepatic plasma membrane glycoproteins (Debray and Montreuil, 1978; Kawasaki and Ashwell, 1976a; Debray *et al.*, 1981), sindbis virus glycopeptide (Burke and Keegstra, 1976), vesticular stomatitis virus G protein (Reading *et al.*, 1978; Etchison *et al.*, 1977), glycopeptide B-3 from calf thymocytes (Kornfeld, 1978), fibronectin (Takasaki *et al.*, 1979) and Semliki virus membranes (Pesonen and Renkonen, 1976).

A pathway for the synthesis of the asparagine-linked carbohydrate structures has been derived from the work of various laboratories with several tissue sources, but is probably applicable to all eucaryotic organisms. The scheme is outlined below with reference to the workers making major contributions to each step in the pathway. In general, the oligosaccharide $(Glc)_3$ $(Man)_9$ $(GlcNAc)_2$ is built up on the lipid intermediate dolichol pyrophosphate by the sequential addition of sugar residues catalysed by specific glycosyl transferases. The G-oligosaccharide, as it has come to be known, is transferred intact to a protein acceptor where subsequent modifications lead to the production of either the high mannose type or the complex type of oligosaccharides found in membrane glycoproteins. The initial work underlying present recognition of the role of polyisoprenoid lipids in the glycosyl transferase reactions was carried out by Leloir and his coworkers (Behrens and Leloir, 1970; Behrens *et al.*, 1971a).

Dolichol is an α saturated polyprenol with 17 to 21 isoprene

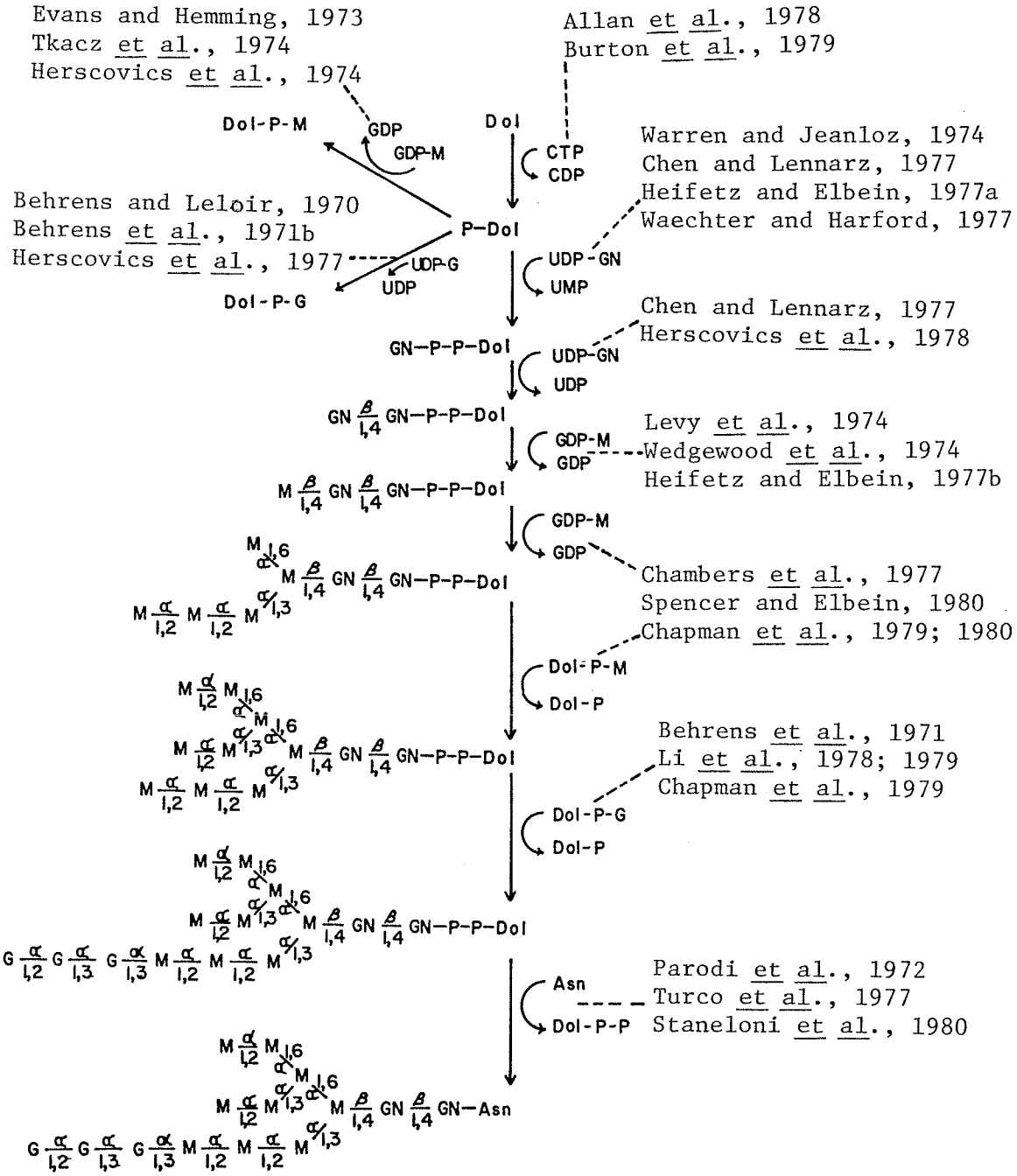
residues synthesized mainly in the outer mitochondrial membrane (Daleo *et al.*, 1977) and is found in the unsaponifiable fraction of animal tissue (Hemming, 1974). Its structure is as follows:

$$H(CH_2\text{-}\underset{CH_3}{C} = CH\text{-}CH_2)_{17-21} -CH_2 -\underset{CH_3}{CH} -CH_2 -CH_2OH$$

Dolichol-linked oligosaccharide synthesis is initiated with the formation of N-acetylglucosaminyl pyrophosphoryl dolichol by the action of N-acetylglucosaminyl transferase. Following attachment of N-acetylglucosamine-1-phosphate to dolichol phosphate, one residue each of N-acetylglucosamine and mannose is added stepwise probably by N-acetylglucosaminyl and mannosyl transferases respectively. Following core trisaccharide formation, addition of mannose occurs via its GDP-mannose derivative to give an oligosaccharide containing five mannose residues. Further addition of mannose and glucose residues occurs via their sugar-phosphoryldolicyl derivatives. The final G-oligosaccharide product has been found to have a similar structure in several different animal tissues (Chambers *et al.*, 1977; Spiro *et al.*, 1976).

Enzymes catalysing some of these sugar transfer reactions have been either solubilized or partly purified. For example, a mannose transferase that is involved in dolichol diphosphate oligosaccharide formation from GDP-mannose has been shown to form a five mannose compound from an exogenous acceptor in which the newly added mannose joins α 1-2 (Schutzback *et al.*, 1980). Also, dolichol phosphate mannose transferase and GlcNAc-1-phosphate transferase activities have been solubilized and measured *in vitro* (Heifetz and Elbein, 1977a). The subcellular fraction in which most of the activities of oligosaccharide biosynthesis

Figure 3: A scheme outlining the biosynthesis of the lipid-linked oligosaccharide intermediates of asparagine-linked glycoproteins and transfer of the completed oligosaccharide to protein.


Evans and Hemming, 1973
Tkacz et al., 1974
Herscovics et al., 1974
Allan et al., 1978
Burton et al., 1979
Dol-P-M
GDP
GDP-M
Dol
CTP
CDP
Warren and Jeanloz, 1974
Chen and Lennarz, 1977
Heifetz and Elbein, 1977a
Waechter and Harford, 1977
Behrens and Leloir, 1970
Behrens et al., 1971b
Herscovics et al., 1977
P-Dol
UDP-G
UDP
Dol-P-G
UDP-GN
UMP
Chen and Lennarz, 1977
Herscovics et al., 1978
GN-P-P-Dol
UDP-GN
UDP
GN β 1,4 GN-P-P-Dol
Levy et al., 1974
Wedgewood et al., 1974
Heifetz and Elbein, 1977b
GDP-M
GDP
M β 1,4 GN β 1,4 GN-P-P-Dol
M α 1,6
M β 1,4 GN β 1,4 GN-P-P-Dol
M α 1,2 M α 1,2 M α 1,3
Chambers et al., 1977
Spencer and Elbein, 1980
Chapman et al., 1979; 1980
Dol-P-M
Dol-P
M α 1,2 M α 1,6
M α 1,3 M α 1,6 M β 1,4 GN β 1,4 GN-P-P-Dol
M α 1,2 M α 1,2 M α 1,3
Behrens et al., 1971
Li et al., 1978; 1979
Chapman et al., 1979
Dol-P-G
Dol-P
M α 1,2 M α 1,6
M α 1,3 M α 1,6 M β 1,4 GN β 1,4 GN-P-P-Dol
G α 1,2 G α 1,3 G α 1,3 M α 1,2 M α 1,2 M α 1,3
Parodi et al., 1972
Turco et al., 1977
Staneloni et al., 1980
Asn
Dol-P-P
M α 1,2 M α 1,6
M α 1,3 M α 1,6 M β 1,4 GN β 1,4 GN-Asn
G α 1,2 G α 1,3 G α 1,3 M α 1,2 M α 1,2 M α 1,3

are predominantly located has been shown to be the rough endoplasmic reticulum (Czichi and Lennarz, 1977). Lipid oligosaccharide formation may occur by a series of coupled reactions on a transmembrane enzyme complex which is sensitive to proteolysis from the cytoplasmic side of the membrane (Hanover and Lennarz, 1982; Snider *et al.*, 1980).

The G-oligosaccharide is transferred to a protein acceptor *en bloc* by an oligosaccharide transferase, but smaller oligosaccharides may be transferred by this enzyme (Chez and Lennarz, 1976, 1977). Such an activity has been solubilized and purified and the transfer to asparagine peptides has been demonstrated (Das and Heath, 1980). R.D. Marshall (1974) examined the amino acid sequence of many glycoproteins with the conclusion that a necessary condition for glycosylation of an asparagine residue is the sequence Asn-X-Thr(ser), but that other factors can be involved. Hart *et al.*, (1979) tested many peptides as acceptors in hen oviduct membranes and confirmed that this sequence, or sequon as it is called, is a necessary but not sufficient determinant for protein glycosylation. The lipid-dependent glycosyl transferase activity has been shown to be concentrated in the rough and smooth microsomal fraction, but also occurs in cytoplasmic membrane vesicles (Idoyaga *et al.*, 1977; Idoyaga *et al.*, 1979; Parodi and Martin-Barrientos, 1977).

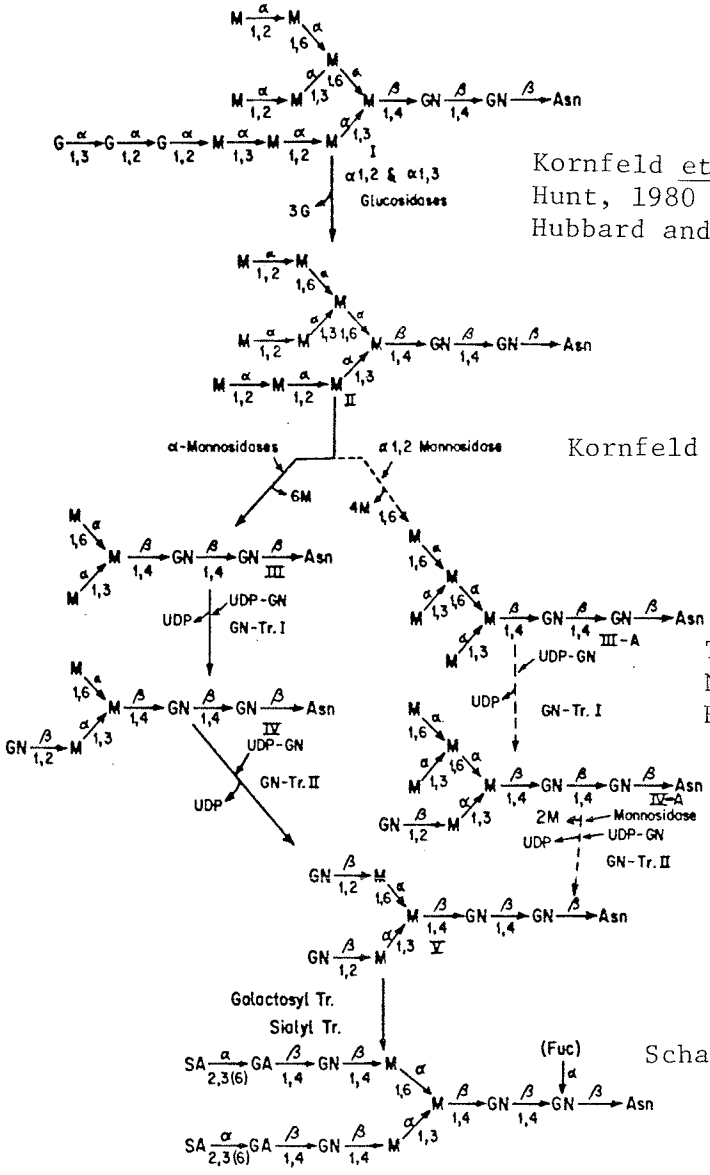
It is generally recognized that messenger RNA translation occurs on membrane bound ribosomes (Wirth *et al.*, 1977), and Blobel has described a "signal sequence" at the amino terminal of the growing peptide chain which facilitates ribosome binding to a receptor on the endoplasmic reticulum with concomitant formation of a channel for passage of the nascent peptide into the lumen of the endoplasmic

reticulum (Blobel and Dobberstein, 1975a,b). Available evidence indicates that transfer of the completed oligosaccharide occurs on nascent polypeptides while they are still attached to ribosomes (Melchers, 1973; Kiely *et al.*, 1976; Rothman and Lodish, 1977), but post-ribosomal glycosylation may be an important mechanism for some proteins (Wolosky and Jamieson, 1980; Bergman and Kuehl, 1979).

Strong evidence that lipid-linked oligosaccharides are the only source of asparagine-linked carbohydrate has come from studies with inhibitors of the pathway (Schwarz and Datema, 1980). Drugs such as tunicamycin, which inhibits an early step in oligosaccharide assembly have been found to simultaneously block glycosylation of asparagine residues (Struck and Lennarz, 1980). Moreover, a temperature-sensitive mutant cell line has been isolated in which the glycoproteins have a reduced asparagine-linked carbohydrate content. Under non-permissive conditions, these cells appear to synthesize a normal lipid-linked oligosaccharide, but the rate of transfer to protein is greatly reduced (Tenner *et al.*, 1977; Tenner and Scheffler, 1979).

After the G-oligosaccharide is transferred to the asparagine residue, processing occurs within minutes with the removal of the three terminal glucose residues. The four exposed α 1-2 linked mannose residues are cleaved following terminal glucose removal, but not until about ten minutes after the oligosaccharide transfer to protein (Hubbard and Robbins, 1979). It must be remembered that while oligosaccharides are being processed, the glycoprotein is being transported through several intracellular compartments. Glucosidases have been prepared from rat liver and hen oviduct that are capable of removing terminal

Figure 4: A scheme outlining processing of oligosaccharide transferred to protein from lipid intermediates, and biosynthesis of complex oligosaccharide of asparagine-linked glycoprotein.



Kornfeld et al., 1978
Hunt, 1980
Hubbard and Robbins, 1979

Kornfeld et al., 1978

Tabas and Kornfeld, 1978
Narasimhan et al., 1977
Harpaz and Schachter, 1980a

Tabas and Kornfeld, 1978
Harpaz and Schachter, 1980b
Narasimhan et al., 1977
Harpaz and Schachter, 1980a

Schachter and Roseman, 1980

glucoses. The rat liver enzymes are integral membrane proteins that appear to be localized on the rough and smooth endoplasmic reticulum cisternal surfaces (Grinna and Robbins, 1979). The hen oviduct glucosidase was highest in the microsomal fraction. This supports the assumption that they process the newly synthesized glycoproteins within the rough endoplasmic reticulum lumen (Elting *et al.*, 1980). The α mannosidase activities of the Golgi membranes are thought to be responsible for cleavage of the α1,2-linked mannoses, leaving a $(Man)_5(GlcNAc)_2$ species containing α1,3 and α1,6 mannoses. This oligosaccharide is typically the smallest found in appreciable amounts in completed glycoproteins (Chapman and Kornfeld, 1979a,b; Cohen and Ballou, 1980). Two α1,2 mannosidase activities have been partially purified from Golgi membranes, and it is assumed that these play a role in the processing steps (Opheim and Touster, 1978; Tabas and Kornfeld, 1979).

After removal of the four α1,2 mannoses, the resulting protein-linked $(Man)_5(GlcNAc)_2$ may be converted to the complex type oligosaccharide. The regulatory events which prescribe limited or extensive processing are not yet understood. Stearic hinderance by a folding polypeptide chain (Hubbard and Ivatt, 1981) or phosphorylation followed by binding to the phosphomannan receptor of the intracellular transport pathway of lysozomal hydrolases (Sly and Fischer, 1982) have been considered to play a role. Interaction with the high mannose oligosaccharide in this fashion may prevent access to the α1,2 mannoses by the Golgi hydrolases, resulting in incomplete processing.

The $(Man)_5(GlcNAc)_2$ oligosaccharide serves as an acceptor for the

activity of GlcNAc transferase I, the first step in converting high mannose oligosaccharides to complex types. After addition of a single GlcNAc residue, a mannosidase activity removes the two "non-core" mannoses. The product is the substrate for several luminal Golgi transferases resulting in elongation of the carbohydrate chains (Carey and Hirschberg, 1981; Kuhn and White, 1975; Fleischer, 1981).

Sugar nucleotides are transported across the membrane of the Golgi apparatus where they are then available as substrates for these luminal glycosyl transferases as shown by specific uptake of nucleotide sugars into the isolated Golgi vesicles (Carey *et al.*, 1980; Deutscher *et al.*, 1982). N-acetylglucosamine transferase II adds a second N-acetylglucosamine residue to the remaining core mannose. A fucosyltransferase can add α1,6-linked fucose to the reducing terminal GlcNAc residue of the core oligosaccharide (Wilson *et al.*, 1976) while a galactosyltransferase forms β1,4 gal linkages to nonreducing terminal GlcNAc residues. The galactosyl transferase has been isolated from a variety of sources and the enzyme's properties have been thoroughly characterized (Roseman and Schacter, 1980).

The linkages between sialic acid and galactose have been found to be primarily α2,3 or α2,6 in a number of glycoproteins. This final step in the elongation process is catalysed by sialyl transferases, one of which has been purified and characterized (Paulson *et al.*, 1977a,b).

There is no evidence for the involvement of a preassembled lipid intermediate for glycosylation in the serine (threonine), N-acetylgalactosamine type of O-glycosidically linked carbohydrate units of glycoproteins. Most knowledge about the overall biosynthetic pathway

is from studies on secretory glycoproteins (mucins) of salivary and mucous glands. Several reviews covering this pathway have been published (Schacter and Roseman 1980; Watkins, 1974, Schacter, 1977). Briefly, the pathway can be summarized by saying the linkage sugar formation as well as the oligosaccharide chain elongation results from stepwise addition of sugar residues directly from their nucleotide derivatives. The first step is N-acetylgalactosamine attachment to serine (threonine) residues by N-acetylgalactosaminyl transferase (McGuire and Roseman, 1967; Hagopion and Eylar, 1968). There is now the possibility of adding either galactose or sialic acid. If the sialyl α2,6-GalNAc disaccharide is formed, further carbohydrate incorporation ceases. If, on the otherhand galactose were incorporated, further carbohydrate residues may be added. The relative abundance of the particular transferase in the tissue is probably the determining factor in the production of a particular oligosaccharide (Carlson *et al.*, 1970).

Analysis of Membrane Glycoprotein Structure and Function by Selection and Characterization of Lectin-Resistant Mutants

Lectins are proteins of non-immune origin, that specifically bind to sugars and/or oligosaccharides without demonstrating enzymatic activity. The interaction of lectins with cell surface carbohydrate groups has provided a powerful tool for investigations concerned with cell membrane architecture. The specificity of such interactions

was initially investigated by inhibiting lectin-induced, cellular agglutination by simple haptene sugars (Goldstein and So, 1965; Lis *et al.*, 1970; Tomita *et al.*, 1970) and gradually led to an appreciation of the great diversity of both source and binding specificities of these proteins (Lis and Sharon, 1973). Several lectins have become commonly applied probes of surface carbohydrate structure because of distinct sugar specificities, availabilities and cytotoxic properties, such as, con A, wheat germ agglutinin (WGA), phytohemaglutinin (PHA) and ricin. The fact that these lectins were cytotoxic to many cultured cells suggested their use as selective agents in obtaining membrane mutants of mammalian cells (Wright, 1973; Ozanne and Lurye, 1974; Hyman *et al.*, 1974). Since the first isolations of cultured cell mutants which failed to interact with specific lectins, many more selections with various lectin-cell combinations have been productive in generating resistant mutants. Literature concerning the biochemistry and cell biology of these cells is now extensive, and has been recently reviewed (Briles, 1982; Wright *et al.*, 1980; Stanley, 1980; Wright, 1979). A summary of the information available from the most well-understood lectin-resistant cell lines is presented here.

One way is which cells may be hypothesized to become resistant to cytotoxic lectins is through modification at the primary target site of the lectin. Ricin and the related two-subunit toxins abrin and modeccin are known to kill cells following an enzymatic modification of the 60S ribosomal subunit by the A chain which penetrates the cell (Olsnes *et al.*, 1975; Olsnes *et al.*, 1978). Mutants with altered interaction between ribosomal subunits and the ricin A chain have not

yet been described.

Primary sites for cytotoxicity of the other lectins mentioned in this section are not understood. Several interactions with cellular membrane-associated metabolic processes have been documented. For example: WGA is known to affect secretion (Dionne and Beaudoin, 1977), metabolic uptake (Li and Kornfeld, 1977) and adenyl cyclase activity (Deschodt-Lanckman *et al.*, 1977); con A is able to mimic the action of insulin on fat cells (Cuatracasas, 1973), increase Golgi membrane galactosyltransferase activity (Young *et al.*, 1976) and it can reduce amino acid transport in cultured cells (Isselbacher, 1972) or inactivate 5' nucleotidase of plasma membrane (Carraway and Carraway, 1976). PHA is able to mimic thrombin activation of platelets (Tollefsen *et al.*, 1974). It is not yet known if any of these effects plays a significant role in the cytotoxicity of the lectin concerned.

Lectin-resistant cell lines that are described in most detail are those in which structural alterations in oligosaccharide moieties of their cell surface glycoproteins preclude, or reduce, binding by the lectin used for selection. The complex asparagine-linked oligosaccharides contain binding sites for several cytotoxic lectins including con A, pea lectin, lentil lectin (LCA), PHA, WGA and ricin. Binding sites usually consist of more than a single sugar residue of the oligosaccharide even though single sugar haptens can compete for binding if present at high concentration (Lis and Sharon, 1973).

Binding specificity of con A involves α-linked mannose residues unsubstituted at C-3, C-4, and C-6 positions (Poretz and Goldstein, 1970). It has been shown that a minimum of two interacting mannose

residues are required to be present in an oligosaccharide before binding will occur to con A immobilized on Sepharose beads. An interacting mannose is either a terminal nonreducing residue or a 2-O-substituted residue (Narasimhan *et al.*, 1979). Complex-type, triantennary structures show no affinity for the lectin, but certain biantennary oligosaccharides containing sialyl, galactosyl-N-acetyl-glucosaminyl branches on 2-O-substituted mannoses in the core structure do interact slightly (Krusius *et al.*, 1976). Presumably, the terminal sialic acid residues prevent a strong interaction. High mannose structures are very good binding substrates for con A because of the large number of terminal and 2-O-substituted mannose residues (Kornfeld and Ferris, 1975).

Pea lectin, PHA and LCA also have binding affinity for complex oligosaccharide core regions since α mannosides are competitive inhibitors. However, additional saccharides essential for interaction with these lectins are known. For example, an α fucose residue linked to the inner core N-acetylglucosamine residue is a key structure determining pea lectin binding (Reitman *et al.*, 1980), while PHA requires outer branch galactose residues for binding (Kornfeld and Kornfeld, 1970). Outer N-acetylglucosamine residues make a significant contribution to the binding of LCA (Kornfeld *et al.*, 1971).

The B chain of the two-subunit lectin ricin binds to terminal galactose residues in either α or β configuration (Olsnes and Pihl, 1980; Baenziger and Fiete, 1979). WGA binds to both sialyl residues and N-acetylglucosamine residues of complex oligosaccharides. Although N-acetylglucosamine is known to be a competitive inhibitor (Nagata

and Burger, 1974), WGA binds very poorly to cells which lack surface sialic acid residues (Bhavanandan and Katlic, 1979). The cross-reactivity of these sugars may be due in part to the similar configurations at the C-2 and C-3 positions (Peters *et al.*, 1979; Monsigny *et al.*, 1980). Some of the O-glycosidically linked oligosaccharides present possible binding sites for WGA because of terminal sialic acid residues, (Bhavanandon *et al.*, 1977; Bhavanandon and Kaltic, 1979), while the desialysed structures are excellent receptors for ricin because of the newly exposed terminal galactose residues (Rosen and Hughes, 1977). Glycolipids also possess potential attachment sites for cytotoxic lectins (Richards *et al.*, 1979; Rosen and Hughes, 1977) although the binding mechanisms involved in these interactions have not been studied extensively. Membrane glycolipids have been shown to interact with WGA (Briles *et al.*, 1977) and ricin (Hughes and Gardas, 1976; Surolia *et al.*, 1975). However, mannose is not a normal constituent of membrane glycolipids and lectins like con A which show high affinity for this membrane component may not interact well with the carbohydrates residues of these membrane lipids (Yogeeswaran *et al.*, 1974; Goldstein and Hayes, 1978).

It should be noted that the interaction of lectins with complex structures such as glycoproteins and glycolipids at the cell surface is an intricate process in which more than one monosaccharide may participate and in which interactions among various receptors and other membrane components play poorly understood roles (Goldstein and Hayes, 1978; Narasimhan *et al.*, 1979;Olsnes and Pihl, 1980; Monsigny *et al.*, 1980; Wright and Ceri, 1977).

Several variant cell lines which bind relatively less lectin at their cell surfaces have been characterized in great detail, concerning the nature of the biochemical alteration which confers lectin-resistance. If assumptions about the biological consequences of a change in gene expression are to be valid, the underlying genetic mechanism(s) responsible for the altered phenotype must be understood. Since the first isolation by Puck and coworkers in 1956 (Puck and Fisher, 1956) of stable variants of cultured somatic cells, much effort has been directed toward elucidating the mechanisms by which phenotypic expression may be altered in a stable fashion. Early concern that epigenetic changes in gene expression would be a major reason for recovering altered phenotypes was expressed by a number of workers. This concern arose from apparent observations indicating no enhancement of mutation rates by treatment with mutagens (Szybalski *et al.*, 1964), the instability of the altered phenotypes (Mezger-Freed, 1971) relatively high mutation rates (Harris, 1971, 1973; Mezger-Freed, 1972), and independence of recessive mutation rates with regard to ploidy (Harris, 1971; Mezger-Freed, 1972). It was argued that if cultured somatic cells could control gene expression in a manner analogous to the programmed changes that occur during embryonic development, then cells with new phenotypes could be selected in culture, yet conform to these initial observations which tended to confirm a non-mutational mechanism of phenotypic alteration.

Positive evidence has been presented to support the existence of formal genetic mechanisms for altered phenotypic expression. Careful analysis of the mutagen-induced frequency of the recessive hprt

(hypoxanthine phosphoribosyl transferase) marker revealed a 25-fold lower rate in tetraploid cell lines when compared to diploid cell lines (Chasin, 1973). Although the mutation rate fell as ploidy increased, it was necessary to hypothesize concomitant segregation-like events playing a role in the appearance of the recessive marker. Some evidence has been presented to show that segregation does occur (Chasin and Urlaub, 1975). Expression of a recessive marker could occur by four mechanisms: chromosome loss; mitotic recombination; gene inactivation or mutation; chromosome loss followed by duplication of the remaining homologue. These possibilities have been tested with cell hybrid lines carrying appropriate marker chromosomes (Campbell and Worton, 1981). Results of this study indicate that loss of a chromosome carrying wild-type alleles, accompanied by duplication of the homologous chromosome carrying the recessive mutant allele was the major (96%) segregation mechanism. In a number of cases (4%), gene inactivation was apparently the means of segregation, but this could have occurred by mechanisms such as: point mutation; small deletions; or epigenetic inactivation. It is therefore, likely that the latter mechanism does not play a significant role in appearance of the new phenotype in these hybrids.

Many recessive phenotypes have been described, incuding temperature-sensitive, auxotrophic, drug-resistant and lectin-resistant cell lines. The existence of such a large variety of loci and the relatively high frequency with which they are isolated in near-diploid cell lines has led to the proposal that large regions of the genome may pre-exist in a hemizygous condition allowing the immediate expression of any recessive

mutations (Siminovitch, 1976). Some regions may also remain functionally diploid however. Selection of two levels of drug resistance at the aprt (adenine phosphoribosyl transferase) and tk (tymidine kinase) loci probably indicates that mutation to the full level of resistance occurs in two distinct steps. The primary step is isolation of a heterozygote with an intermediate enzyme level, followed by an independent selection of a homozygote lacking the enzyme activities (Clive *et al.*, 1972; Jones and Sargent, 1974).

Siminovitch (1976) has summarized the available information for some well-studied somatic cell mutants with the conclusion that these arise by genetic changes. Among the criteria used were: stability of the new phenotype in long-term culture; increased mutation frequency in the presence of mutagens, demonstration of reversion, localization to a chromosome and evidence for change in the structure of the protein affected. Although a non-mutational mechanism of "gene inactivation" or "gene silence" is still a viable concept and may play an interesting role in the selection of variant phenotypes in some genetic systems (Milman *et al.*, 1976; Morrow, 1977; Bradley, 1979; Harris, 1982), it is apparent that most mutant selection procedures studied in detail give rise to altered cells with genetic properties satisfying the majority of criteria for somatic cell mutants.

Lectin-resistant variants have made significant contributions to the understanding of the biosynthetic pathway of Asn-linked oligosaccharides. The first mutant to be shown to have a defective step in the biosynthesis of normal oligosaccharides were Chinese hamster ovary cell lines selected for resistance to any one of the lectins PHA, WGA,

LCA or ricin isolated independently in three laboratories (Gottlieb *et al.*, 1974; Stanley *et al.*, 1975a,b; Meager *et al.*, 1975). Each exhibits a similarly complex phenotype including loss of cell surface lectin receptors and cross-resistance to the other lectins listed above, which results from the loss of an N-acetylglucosaminyl transferase activity (Stanley *et al.*, 1975c; Meager, 1976; Gottlieb *et al.*, 1975). The N-acetylglucosaminyl transferase activity which was missing in these cell lines appeared to be one of two activities present in the parental lines and was capable of transferring N-acetylglucosamine to mannose residues in the outer branches of asparagine-linked oligosaccharide groups (Gottlieb *et al.*, 1975; Stanley *et al.*, 1975c). Structurally defined substrates were used by Narasimhan *et al.*, (1977) to define the specificities of the GlcNAc transferase activities in wild-type and resistant cells. It was observed that the variant cells lacked the activity specific for the N-acetylglucosamine addition to terminal mannose residues linked α1,3 to the β-linked core mannose (this activity is often referred to as N-acetylglucosamine transferase I). The N-acetylglucosaminyl transferase activity which was not diminished, acted on core structures bearing an N-acetylglucosaminyl residue on the mannose residue linked by an α1,3 glycosidic bond (referred to as N-acetylglucosaminyl transferase II). Subsequently, it was noted that the processing pathway in the variants was deficient and led to accumulation of a five-mannose structure in which the mannose residue linked α1,6 to the β-linked mannose retained both α1,3 and α1,6-linked mannose residues (Li and Kornfeld, 1978; Robertson *et al.*, 1978). That the missing N-acetylglucosamine transferase

activity was the required signal for complete processing was demonstrated by Tabas and Kornfeld (1978) who found a highly specific α mannosidase activity which removed the two outer mannose residues only after the action of N-acetylglucosaminyl transferase I.

It is clear that mutants of this phenotype have made a major contribution to understanding certain steps in the assembly of the complex oligosaccharide chains of glycoproteins. A similar contribution to steps in the biosynthesis of high mannose oligosaccharides was made by Trowbridge and his colleagues. A mouse lymphoma cell variant defective in Thy 1 antigen (T25 glycoprotein) biosynthesis was shown to possess a pleiotropic glycosylation defect resulting in a reduced ability of the cell surfaces to bind con A (Trowbridge *et al.*, 1978b). Detailed structural studies of the lipid-linked oligosaccharides which accumulate in these mutants revealed a five-mannose structure in which the α1,6 core mannose is terminal, bearing neither α1,6 nor α1,3-linked mannose residues (Trowbridge and Hyman, 1979; Chapman *et al.*, 1979b). This structure can be glucosylated and transferred directly to nascent protein (Chapman *et al.*, 1979, 1980) and subsequently processed to structurally normal oligosaccharides (Kornfeld *et al.*, 1979). The defect in this variant has been shown to be an inability to synthesize dolichol-P-mannose (Chapman *et al.*, 1980), consequently the specific mannosyl transferase(s) responsible for addition of the outer α-linked mannoses on the α1,6-linked core mannose cannot complete the normal lipid-linked G-oligosaccharide. The extent of the contribution of dolichol-P-mannose during high mannose oligosaccharide biosynthesis was thus shown to be limited to four specific mannose residues, while

the remaining part of the structure could be assumed to be built up by transfer from GDP-mannose alone.

A double-variant Chinese hamster ovary cell line has been selected resistant to first PHA, then con A (Stanley, 1975b; Stanley and Siminovitch, 1977). The N-acetylglucosaminyl transferase deficiency which normally would lead to the accumulation of a five-mannose structure containing both α1,3 and α1,6 mannose residues linked to the α1,6 core mannose, in the double variant was shown to lead to accumulation of a four-mannose structure in which the α1,6-linked, unprocessed mannose was absent (Hunt, 1980a; Robertson *et al.*, 1978). Moreover, the largest lipid-linked oligosaccharide detected was a seven-mannose structure (Hunt, 1980a,b), presumably because the missing α1,6-linked mannose precluded addition of the non-reducing, outer α1,2-linked mannose on that branch of the oligosaccharide. The biochemical basis for the oligosaccharide truncation in these cells has not yet been established.

Many other lectin-resistant cell lines have also been selected which as yet are not understood well enough to allow a positive identification of the lesion responsible for reduced lectin binding. Several Chinese hamster ovary cell lines resistant to con A have been selected independently in three different laboratories which have a modest, two-to three-fold resistance and an unusual temperature-sensitive phenotype (Wright, 1975; Wright and Ceri, 1977a; Wright *et al.*, 1979; Cifone and Baker, 1976; Cifone *et al.*, 1979; Krag *et al.*, 1977; Briles *et al.*, 1978). It is assumed that all of these isolates represent a single class of mutant (Briles, 1982). The normal VSV G-protein

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synthesized by these variants apparently has oligosaccharides which are not truncated, but are fewer in number than those in the normal protein (Briles, 1982). Defects in the biosynthesis of lipid-linked oligosaccharide precursors which may be able to account for this loss of oligosaccharides have been observed (Krag *et al.*, 1977; Wright *et al.*, 1979) and the defect may be attributed to the loss of an ability to transfer glucose to lipid-linked oligosaccharides (Krag, 1979). Since non-glucosylated oligosaccharide is known to be a poor substrate for transfer to nascent polypeptide, some normally glycosylated proteins may lack a full complement of oligosaccharide structures. It has been shown that some non-glycosylated glycoproteins are more sensitive to denaturation at elevated temperature (Gibson *et al.*, 1978). It is tempting to speculate that a similar mechanism in physiologically important peptide may account for the cellular sensitivity to elevated temperature displayed by con A-resistant CHO cell lines.

A second class of WGA-resistant mutants has been described which is about ten-fold resistant to the lectin and concomittantly hypersensitive to ricin, PHA and LCA (Briles *et al.*, 1976, 1977; Stanley *et al.*, 1980). The membrane glycoproteins and glycolipids contain reduced amounts of sialic acid; however, these cells contain the appropriate nucleotide sugars, glycosyl transferases, glycoproteins and glycolipid acceptors. The lesion in these variant cells may affect the *in vivo* organization of the multi-glycosyl transferase system resulting in under-sialylated complex oligosaccharides with exposure of penultimate galactose residues as the terminal sugars.

During the selection and biochemical characterization of lectin-resistant cell lines, it became apparent that alterations in glycoprotein biosynthesis and processing pathways often had significant effects on cell surface properties. For example, early studies of variant cell lines expressing altered interactions with con A showed that a large number of SV-40 transformed 3T3 cells surviving short exposures to high concentrations of the lectin could be isolated that also reverted to an untransformed state when measured by several *in vitro* tests for transformation, such as saturation density, agglutinability by con A and cellular morphology (Culp and Black, 1972; Ozanne and Sambrook, 1971). Also Chinese hamster ovary cells selected for the ability to grow in normally cytotoxic concentrations of con A were shown to possess a very interesting pleiotropic phenotype, which included changes in growth and membrane-associated properties (Wright, 1973; Siminovitch *et al.*, 1972). It was also observed in these initial studies that the total number of lectin receptors on the variant and wild type cells was unaltered if measured at saturating concentrations of the lectin. However, these early binding experiments were carried out at room temperature where it is now known that pinocytosis can mask subtle differences in lectin binding with many cell lines (Noonan and Burger, 1973; Wright and Ceri, 1977b). It was clear, however, from these pioneering studies that membrane variants resistant to the cytotoxic effects of lectins could be selected, and that important changes in the biological activities of the cells could occur.

In the ensuing decade since the first of these transformation-revertant selections, more rigorous selections of cells produced more

highly resistant, oligosaccharide-defective lines of cells which were also often observed to have alterations in important cellular events in which the membrane was assumed to play a role. Cellular adhesiveness to the substratum has been shown to be reduced in a number of these cell lines when compared with their parental lines, for example: PHA-resistant (Juliano, 1978), ricin-resistant (Edwards *et al.*, 1976), WGA-resistant (Briles *et al.*, 1977) and con A-resistant (Ceri and Wright, 1977a) lines can all be released more easily by trypsin and/or EDTA treatment. The temperature-sensitive nature of the con A-resistant phenotype in Chinese hamster ovary cells made possible the selection of lectin-sensitive revertants and therefore a more rigorous examination of the relation between lectin resistance and altered adhesiveness. Investigations performed with various con A-resistant, revertant and sensitive cell lines showed that a direct correlation existed between the con A-resistant property and an altered phenotype which included decreased adhesiveness in addition to several other altered membrane-related properties (Ceri and Wright, 1978a).

Lectin-resistant variants have been used to investigate certain details of virus-cell interaction. Stanley and Siminovitch (1977) have shown that Sendai virus is unable to induce fusion of the sialic acid-deficient variant $Wga^{R}II$, presumably because of inappropriate cellular receptor structures. In a similar fashion, lectin-resistant cells missing N-acetylglucosamine transferase I and therefore accumulating unprocessed complex oligosaccharide structures, cannot be fused with Newcastle's disease virus (Poles and Gallaher, 1979). It is apparent then that appropriate cell surface glycoprotein receptors are

required for normal virus-cell interactions.

Finally, a ricin-resistant mouse lymphoma cell line selected by repeated exposure to increasing ricin concentrations is lacking a 35,000 m.w. protein which is present in the parental cells (Nicolson *et al.*, 1976; Robbins *et al.*, 1977). A second, ricin-binding protein is decreased from 80,000 m.w. in the wild-type line to 70,000 in the variant. The variant exhibits a decreased capacity to endocytose surface bound ricin, suggesting that the altered membrane proteins participate in this process. It has been suggested by Olsnes and Pihl (1978) and Pappenheimer (1978) that the toxic subunit of ricin may gain entry into the cytosol by a specific cellular mechanism which normally mediates the cytosolic uptake of physiologically important molecular messengers. It could be that the proteins altered in the ricin-resistant mouse lymphoma variants are components of that system. It is worth noting that con A-resistance in hamster cells (Ceri and Wright, 1978b) and in mouse lymphoma cells (Trowbridge *et al.*, 1978a,b) can lead to changes in specific glycoproteins as opposed to general changes in all surface glycoproteins. In these cases however, no functions are known for the glycoproteins of interest, therefore, it is impossible to predict what cellular functions are directly affected by them.

Studies of Myogenesis *In vitro*

Muscle development (myogenesis) refers to the development of

proliferating embryonic muscle cells (myoblasts) into terminally differentiated, mature muscle fibres made up of fused multinucleated myotubes (myofibres). Myogenesis *in vivo* is observed primarily during embryological development, but may also occur during regeneration of damaged muscle in adult animals. In vertebrates, myoblasts are committed mesodermal cells derived from the inner wall of somites (the myotome) and from mesenchyme tissue of the lateral plate mesoderm in the early embryo. These cells become recognizable as myogenic after their rearrangement into longitudinally elongated masses. Subsequent cellular migrations within the embryo carry the myogenic cells to locations in such structures as limb buds, where additional growth and differentiation takes place (Balinsky, 1970). Following birth, considerable differentiation still occurs in the morphology and physiology of the muscle fibres as they increase in size. This maturation includes an increase in the number of myonuclei within myotubes which depends on continued myoblast proliferation and their fusion with differentiating myotubes (Moss and Leblond, 1970).

The cells with myogenic potential that remain in mature muscle are known as "satellite cells" because of their close association with the myofibre sarcolemma (muscle cell plasma membrane). These cells are presumably trapped during embryonic development within the basement lamina of the fibres where they serve as a reserve myogenic precursor cell population. In response to muscle damage, satellite cells are activated to proliferate and repopulate the basement laminar sheath of the degenerated fibre, subsequently forming a new fibre (Allbrook, 1981; Bischoff, 1979).

Primary Myoblasts as Models of Myogenesis

The choice of muscle tissue as a model developmental system in which to study cell surface structure-function relationships is a good one. The transition from mononucleated myoblast to multinucleated myotube is a striking event from the morphological standpoint alone. Clearly, the surface membrane is intimately involved in the mechanism by which myoblasts recognize, align with and finally fuse with the growing myotube. The possibility also exists that the cytoplasmic membrane may directly mediate the original event responsible for activating the cellular components involved in terminal differentiation and one can hypothesize that surface carbohydrate structures might be part of this process.

The practical aspects of studying developing muscle tissue *in vitro* also make this an attractive choice. Mononucleated spindle-shaped myoblasts from many species can be obtained in large amounts by dissociating embryonic muscle tissue into single cell suspension. Such cells are able to undergo the terminal stages of myogenic differentiation in culture, forming the morphologically distinct cross-striated multinucleated myotube capable of spontaneous contraction. Moreover, the myoblasts may be grown as secondary cultures to obtain not only large amounts of developmentally competant cells, but clonal populations, facilitating further experimental analysis.

Embryonic myoblasts have been routinely prepared from the skeletal muscle of chicken (Oh, 1975), quail (Konigsberg, 1971), mouse (Yaffe and Saxel, 1977a), rat (Shainberg *et al.*, 1971) and human (Hauska *et al.*,

1979) muscle explants by treatment with trypsin or collagenase until they are dissociated to the single cell level, followed by plating in tissue culture medium supplemented with serum and often embryo extract (Konigsberg, 1979). Unfortunately, fibroblasts may heavily contaminate such preparations of myoblasts. The stelate shape of the fibroblast usually distinguishes it from the spindle-shaped myoblast. A "selective serial passage" technique for contaminated populations was developed to deplete the numbers of fibroblasts by virtue of their more rapid attachment to the plastic plate surface (Richler and Yaffe, 1970). Floating myoblasts are recovered before they too have attached and are replated in dishes coated with collagen to promote attachement and differentiation. With such procedures it is now possible to obtain gram amounts of myoblasts that may be 90% pure.

When plated at low cell density in culture medium, the myoblasts well grown exponentially with a generation time of from 12 to 20 hours depending on the species and culture conditions until a monolayer of about 10^5 cells/cm^2 has been formed. If the cells are not re-trypsinized and diluted at this time, myogenesis will commence with the production of multinucleated myotubes.

Myoblast fusion *in vitro* has been observed in great detail by many authors at both the level of light and electron microscopy. Fusion occurs between adjacent myoblasts in an end-to-end or end-to-side fashion (Konigsberg *et al.*, 1978). Cinematography has shown directly that mononucleated myoblasts form the myotubes by membrane fusion (Capers, 1960). Mixtures of myoblasts with unlabelled nuclei and nuclei labelled with [^{3}H] thymidine formed myotubes containing both

labelled and unlabelled nuclei, confirming that fushion led to multi-nucleation (Yaffe and Feldman, 1965). No DNA synthesis could be shown during myotube growth, which eliminated the possibility of nuclear replication making a contribution to the process (Friedlander *et al.*, 1978; Yaffe and Feldman, 1965). It was noted that muscle-specific surface properties extend across wide taxonomic differences since many types of interspecies hybrid myotubes could be formed in culture (Yaffe and Feldman, 1965).

Filopodial contacts (Chiquet *et al.*, 1975) may facilitate the initial cytoplasmic bridges formed before complete membrane fusion (Lipton and Konigsberg, 1972) in particle-free areas of the membrane (Kalderon and Gilula, 1979). Gap junctions are known to form prior to fusion, but their contribution to the fusion of the lipid bilayers is not clear (Kalderon *et al.*, 1977). Microtubules and developing microfilaments are predominantly oriented longitudinally just beneath the plasma membrane in myoblasts (Lipton, 1977; Warren, 1974), but it is not known if this arrangement leads to the organization of actin and myosin filaments into sarcomeres later in development. The Golgi apparatus is well developed in myoblasts (Lipton, 1977) and myotubes (Fukuda *et al.*, 1976) presumably reflecting its importance in glycosylation and secretory transport during development.

For several days after fusion the muscle cultures will maintain their structural integrity, but then begin to undergo muscle fibre atrophy and degeneration, with loss of spontaneous contractions, cross-striations and fibre size (Markelonis and Oh, 1979). Further improvements in culture technique will be necessary to allow experiments

which might demand the long-term maintenance of the muscle fibres in culture.

Two hypotheses have been formed to explain the transition of proliferating myoblasts to the differentiated state. Holtzer and his co-workers have postulated that myogenic differentiation requires an event termed a "quantal mitosis" during which modifications are made in the genetic program needed to initiate a new developmental pathway. The pathway is triggered during the S phase of the "quantal" mitosis and sets in motion the metabolic steps required for subsequent fusion and biochemical differentiation (Bischoff and Holtzer, 1969; Holtzer *et al.*, 1972; Dienstman and Holtzer, 1977).

On the other hand, Konigsberg and collaboraters have provided strong evidence for a probablistic concept of commitment to differentiation in which no DNA synthesis is required. According to these investigators, the probability that a cell will fuse is directly correlated to the length of the G1 period of the cell cycle (Buckley and Konigsberg, 1974; Konigsberg *et al.*, 1978). More work is obviously required to settle these contradicting claims, but because of different myoblast systems and experimental protocols, direct comparison of the findings is made difficult.

During myogenesis *in vitro* many intracellular changes occur in the concentrations of specialized proteins characteristic of mature muscle. Some of the muscle specific proteins are not present initially in the myoblasts and appear only later during differentiation. These increases have been particularly useful as "markers" for the differentiation process during experimental analysis. Some of the more

common biochemical changes that have been used are: actin (Storti *et al.*, 1976), myosin (Patterson and Strohman, 1972), and acetyl choline receptor (Fambrough and Rash, 1971). A complete list of biochemical alterations occurring during differentiation has been compiled by Pearson (1980). However, because no detailed genetic and biochemical analyses have been made, many of these changes may result from simply saturating cell densities and the cessation of growth.

Permanent Myogenic Cell Lines as Models of Myogenesis

A major difficulty encountered when working with the primary myoblast cultures was the cessation of proliferation after 50 to 60 doublings. The process of senescence originally described by Hayflick (1965) undoubtedly accounts for the limited life span of the myoblasts. Observations on cell doubling capacity indicate that human muscle colony-forming cells taken from donors of 70 years of age are capable of only about 30 doublings, presumably reflecting the fact that the adult myoblasts have normally undergone more cell cycles *in vivo* and are closer to their maximal number of mitoses (Hauschka *et al.*, 1979).

Among the senescent cells at high passage numbers, cells which had not lost proliferative capacity could occasionally be observed to form colonies (Yaffe, 1968; Richler and Yaffe, 1970; Hauschka *et al.*, 1979). When examined for differentiation properties, many colonies manifested normal muscle fibre formation and a lack of DNA synthesis in those structures. Both the rat and mouse permanent lines that were

selected have not lost the capacity to differentiate after thousands of passages in culture. The L6 and L8 rat myogenic lines of Yaffe have particularly good plating efficiency and fusion potential. They are now widely distributed and have found many applications in myogenesis research. A permanent myogenic cell line (T984) was also isolated from a mouse teratocarcinoma (Gearhart and Mintz, 1975; Jacob *et al.*, 1978). Prolific growth is obtained in normal culture media in the absence of collagen or feeder layers, and at confluence myogenic differentiation may proceed with production of a very dense network of contracting fibres. Periodic cloning of a population selected for the ability to differentiate is necessary to remove non-fusing variants that may accumulate during routine culture.

Fusion of L6 myoblasts into multinucleated myotubes is associated with cessation of DNA synthesis, as was known for primary myoblasts (Yaffe, 1968). However, the myotube formation was not as synchronous - the increase in size and number of myotubes extends for several days past confluence of the monolayer. The question of a requirement for a "quantal cell cycle" that has been raised for myogenesis in primary cultures has been investigated using L6 subclones, again with conflicting results. Nadal-Ginard (1978) determined that when the myoblasts are arrested in G_1 stage of the cell cycle by high cell densities and DNA synthesis inhibitors, the probability of a myoblast beginning the differentiation program and irreversibly ceasing proliferation was a stochastic process. Contrary to this finding, Delain *et al.*, (1981) using serum deprivation to inhibit DNA synthesis have shown that it is required before withdrawal from the cell cycle and initiation of

differentiation. Obviously, more work will be required to resolve this question.

The extent to which the myogenic phenotype is retained by the continuous cell lines is even more evident when one considers the biochemical differentiation that is known to occur concomitant to fusion (Pearson, 1980). Among the more commonly measured biochemical parameters of differentiation are creatine kinase (Shainberg *et al.*, 1971), adenylate kinase, glycogen phosphorylase and acetylcholine receptor (Patrick *et al.*, 1972). Together with the conveniences of homogeneity of cell type, developmental stage, high plating efficiency and *in vitro* proliferative capacity, these characters have lead to an increasing use of the myoblast lines as models for the myogenic process. Clearly, some reservations are required about the model of differentiation because of the "spontaneous transformant" nature of the origin of, for example L6 and L8 lines. The primary myoblasts from which L6 was selected were treated with the tumor inducing agent methylcholanthrene,although L8 was isolated without mutagen treatment. Nevertheless, both these lines express the transformed phenotype (Bignami, 1982). Retention of the capacity to differentiate is therefore not indicative of normality. It is conceivable that expression of transformation-associated traits during the proliferative phase might cause the myogenic lines to have adopted new decisional mechanisms necessary to bring about cessation of proliferation and transition to the differentiation phase.

Experimental Analyses of Myogenesis *In Vitro*

The literature is replete with experiments employing exogenously applied biochemical agents or other physical treatments as probes of the myogenic process with the idea that if their mode of action could be determined, they might point directly to the molecular processes essential for normal myogenesis. Several have already been mentioned as inhibitors of DNA synthesis. A fundamental understanding of any mechanisms underlying cell cycle withdrawal, commitment to differentiation or production of the differentiated state by myoblastic cells has not yet arisen from experiments of this type. The difficulty arises directly from the lack of a conceptual framework for the myogenic mechanism from which specific probes might be rationally chosen. Nevertheless, a number of studies have pointed to the importance of the myoblast cytoplasmic membrane as a crucial regulatory component of the myogenic process.

Macromolecular effectors applied to myoblasts in culture would be expected to interact primarily with the surface membrane. Several proteins have been studied that are likely to be important for normal muscle development *in vivo*. For example, collagen coated substrate was found by Hauschka and Konigsberg (1966) to replace the requirement of the fusion process in primary chick myoblasts for medium conditioned by exposure for several days to a dense population of cells. One of the fusion-promoting factors in chick embryo extract that cannot be filtered with a 5×10^{-4} m.w. cut-off ultrafiltration membrane was shown to be collagen (de la Haba *et al.*, 1975). The major collagen

synthesized by embryonic chick muscle was found to be type I collagen, but types I, II, III and IV collagens were equally effective in promoting myoblast fusion (Ketley *et al.*, 1976). A substratum coated with the molecule appears to be essential for enhancement of myotube formation and it thus may act by stabilizing the membrane interactions that lead to fusion. It is known that the electrical charge of the substratum may modulate differentiation (Wahrmann *et al.*, 1981), again suggesting that cell membrane interactions may be altered. L6 myoblasts have been shown to synthesize and secrete their own collagen (Schubert *et al.*, 1973) which probably accounts for the fact that preconditioned media or chick embryo extract are not required for differentiation in this line (Florini and Roberts, 1979). Addition of purified fibronectin, a major cell surface protein probably involved in cellular attachment to the substratum, blocks fusion in L6 myoblasts and permits an increase in cell number. Removal of this protein at critical times can stimulate fusion. Presumably fibronectin prepares cells to fuse but it must be reduced during differentiation otherwise no relief from the inhibitory effect is seen (Podleski *et al.*, 1979a).

Insulin, when added to primary avian myoblast cultures (de la Haba, 1966, 1968) or the rat L6 myoblast line (Mandel and Pearson, 1974) is able to stimulate myogenesis. Although this hormone is able to stimulate many other activities of muscle metabolism, the regulation of intracellular cyclic nucleotide concentration (cAMP and cGMP) is attractive as an effect to account for myogenic stimulation (Ball and Sanwal, 1980), in view of the regulation of adenyl cyclase during differentiation (Wahrmann *et al.*, 1973a), and the reversible inhibition

of myogenesis shown by cAMP, dibutyryl cAMP and theophylline (Wahrman *et al.*, 1973b), or the stimulation observed by treatment with β adrenergic catecholamines (Curtis and Zalin, 1981).

Inductive effects of nerve tissue on muscle development have been observed for many years (Gutmann, 1976). Neurotrophic influences have been predicted to play a role in this influence and recently, a nerve protein has been purified that can influence the maturation and long-term maintenance of cultured muscle fibres in a manner that resembles some of the components of enhanced development after innervation (Markelonis and Oh, 1979). The mechanisms that might play a role in the enhanced development have not yet been described.

Other reports have appeared in the literature describing the proteinaceous material present in conditioned medium that stimulates fusion of developing myoblasts (Doering and Fischmann, 1977; Delain *et al.*, 1981). Further characterization of the interaction of these protein effectors with the myoblast surface membrane should lead to new insights into the muscle differentiation regulatory responses.

Inhibitors of myogenesis acting on the myoblast cell surface have also been instrumental in supplying information relating to the participation of membrane interactions during differentiation. Under appropriate conditions, myoblast fusion is inhibited by lectins such as con A, abrin, wheat germ agglutinin or *Lens culinaris* agglutinin (Den *et al.*, 1975; Burstein and Shainberg, 1979). This effect may be due to a restriction of the mobility of glycoprotein receptors within the myoblast membrane (Sandra *et al.*, 1977). Some interesting studies with a β-D-galactoside specific lectin, found in embryonic chick

pectoral muscle and in skeletal muscle cultures, indicate that this lectin is present in high concentrations before fusion, leading to the speculation that it has a direct role in the differentiation process by promoting intercellular adhesion (Podleski *et al.*, 1979b; Nowak *et al.*, 1977; Franklin *et al.*, 1980). Further support for this idea comes from observations on the ability of this lectin to block cultured chick myoblast fusion, presumably by covering the lactose containing receptors on adjacent cells (MacBride and Przybylski, 1980). Some conflicting data concerning the exact role played by endogenous muscle lectin activities is apparent however, when it is noted that primary cultures of chicken (Den *et al.*, 1976) and rat skeletal (Den and Malinzak, 1977) muscle are unaffected by the hapten inhibitor thiodigalactoside, but that rat muscle cell lines L6 and L8 are fusion inhibited (Den and Malinzak, 1977; Gartner and Podleski, 1975). Further controversy over a specific role in myogenesis is apparent when reports of the predominantly intracellular location (Beyer *et al.*, 1979) and wide tissue-type distribution of this lectin are considered (Kobiler *et al.*, 1978).

Recently, it has been observed that inhibition of protein glycosylation by the antibiotic tunicamycin prevents myotube formation, suggesting that some glycoproteins not needed for proliferation, are essential for myoblast fusion (Gilfix and Sanwal, 1980). Fusion inhibition reversal by protease inhibitors suggests that the oligosaccharides of the glycoproteins important to differentiation act to stabilize the protein moiety against proteolytic degradation (Olden *et al.*, 1981). The exact nature of the molecules that are involved

remains to be characterized.

Treatment of the cell surface with glycosidases such as neuraminidase has led to conflicting reports regarding the effect on fusion of myoblast membranes (Dahl *et al.*, 1978; Knudsen and Horwitz, 1978). These same studies however, point to the importance of proteinaceous membrane components in the membrane recognition and fusion mechanism. In this connection, it is interesting to note that cell surface labelling studies have shown increased synthesis and accumulation of a fucose-containing surface glycoprotein coincident with the rapid phase of fusion in primary chicken myoblasts (Cates and Holland, 1978). In the L6 myoblast line, differences have been shown in the size distribution and carbohydrate composition of externally exposed glycopeptides obtained at different stages of differentiation, suggesting a regulatory linkage with the myogenic process (Winnand and Luzzati, 1975; Ng, 1980; Wahrmann *et al.*, 1980; Senechal *et al.*, 1982; Cossu *et al.*, 1981).

Inhibition of myogenesis by BUdR (bromodeoxyuridine) was originally reported by Coleman *et al.*, (1969) and was thought to result from incorporation of the nucleoside analogue into DNA with a subsequent inability of the analogue-substituted DNA to act as an efficient template for normal transcription during differentiation (Bischoff and Holtzer, 1970). Reversal of the inhibition could be achieved by simultaneous addition of deoxycytidine (CdR) to the medium to reduce the BUdR-dependent reduction of CdR synthesis but continuing to allow DNA replication with extensive analogue incorporation, leading Rogers *et al.*, (1975) to suggest that the actual inhibition site may be the one or more glycosyl transferases which require activated nucleotide-

sugars and are responsible for synthesizing fusion-specific glycoproteins or glycolipids.

In summary, the studies of myogenesis, using a wide spectrum of biochemical approaches have underscored the important regulatory function played by the cytoplasmic membrane during the myogenic process. Few details of the structural requirements for these functions have yet been revealed by presently available biochemical techniques, but rather only an understanding of the ease with which the normal myogenic phenotype can be disrupted *in vitro* and is probably indicative of a complex web of interdependent structures and events leading from uncommitted myoblasts to the final, terminally differentiated myofibre. The solution to this situation may reside in the isolation and characterization of mutations at each step in these regulatory pathways. Based on the information available from the preceding biochemical studies, useful selection schemes could be designed to generate mutant cells that are affected in muscle development. By genetic and biochemical comparisons of mutant and wild-type myoblasts, it might be then possible to devise clear-cut models for the molecular interactions controlling myogenesis.

Somatic Cell Genetics as a Tool in the Analysis of Myogenesis

Myoblast cell lines such as L6 and L8 offer a convenient model system in which to isolate mutations and to study the effect on the myogenic phenotype of the alterations in cellular structures and functions

which result from a heritable change in the DNA base composition. The caveat to be considered is that these cell lines may only superficially represent the myogenic phenotype expressed by normal diploid skeletal myoblasts. However, since individual myoblasts divide by asexual reproduction and form discrete colonies, genetically uniform populations can be obtained and eventually grown in large batch cultures. The permanent nature of the lines also ensures that loss of growth potential displayed by normal diploid somatic cells after 50 to 80 doublings would not complicate analysis of a mutation once it has been selected.

The first "mutant" myoblast cell lines to be described were selected visually on the basis of their inability to differentiate, after nitrosoguanidine mutagenesis (Loomis *et al.*, 1973). The frequency of variants recovered from the surviving population was surprisingly high (12 of 34 clones analysed) and many of the variants were observed to be temperature sensitive. Other spontaneous fusion-defective mutants have since been isolated; some apparently are conditional (Yaffe and Saxel, 1977b) while others appear to have attained a transformed phenotype (Kaufman and Parks, 1977; Kaufman *et al.*, 1980). Several retain partial expression of the muscle specific phenotype as measured by creatine kinase and adenylate kinase activities (Tarikas and Schubert, 1974; Pearson, 1980). In all of these cases, the molecular nature of the defect in myogenesis is unknown. The altered phenotype could be due to a mutation in any one of a large number of different gene products, or alternatively, an epigenetic attenuation of the ability to differentiate may be induced in culture. Either

mechanism might account for the high frequency with which such "mutations" are observed. The spontaneous rate of mutation to the myogenic-defective phenotype has been estimated to be as high as 10^{-4}/cell/generation (Pearson, 1980), and indicates that care must be taken in the suggestion of a relation between a known genetic defect and defective myogenesis.

Selection of mutant myoblast cell lines with more defined biochemical lesions is only now in its beginnings. One interesting study has been described by Crerar *et al.*, (1977), in which a mutant myoblast line selected for resistance to the RNA polymerase inhibitor α amanatin was shown to contain two RNA polymerase II activities (one resistant and one sensitive) and that the resistant enzyme became the dominant form within cells in the presence of drug. A subset of this class of mutant exhibited an α amanitin-dependent defect in myogenesis, suggesting that Ama^R mutations result in a pleiotropic defect in myogenesis as a result of altered patterns of transcription by the mutant form of RNA polymerase II (Pearson, 1980).

Azacytidine-resistant mutants which are unable to differentiate were used in a study which examined developmental changes in gangliosides during differentiation (Whatley *et al.*, 1976). The ganglioside GD_{1a} was shown to transiently increase three-fold just prior to wild-type L6 myoblast fusion and then return to basal levels once myotubes had formed. The drug resistant, non-fusing line did not synthesize GD_{1a} at any time during cell culture. It was suggested that GD_{1a} participates in the fusion process by destablizing the membrane lipid bilayer in local areas of high concentration. How

the drug resistant phenotype directly affects ganglioside metabolism in this line remains unknown.

Selection of con A-resistant L6 myoblast variants has been used to test the hypothesis that cell surface glycoproteins play a role in myogenesis (Parfett *et al.*, 1981). A tight correlation among the properties of lectin-resistance, altered glycoprotein biosynthesis and defective differentiation suggested that mannose-containing glycoproteins may play a special role during myogenesis, and that cells with the altered phenotype are unable to initiate the developmental program.

More recently, our laboratory has initiated work designed to determine what role might be played by the enzyme ribonucleotide reductase. Mutants resistant to hydroxyurea (a potent inhibitor of the enzyme) that are consistantly altered or defective with regard to myogenesis, have been selected from wild-type L6 populations leading to speculation that structural or regulatory changes affecting deoxy-nucleotide synthesis have a direct effect on the ability of myoblasts to undergo the developmental program (Creasey *et al.*, 1982; Wright *et al.*, 1982).

In addition, it is worth noting that several mutant myoblast lines resistant to azaguanine (Luzzati, 1974; Dufresne *et al.*, 1976, Rogers *et al.*, 1978) BUdR (Merrill and Hauschka, 1978) and ouabain (Luzzati . 1974) have been isolated. The myogenic phenotype has been retained by these cell lines because they possess changes in biochemical pathways or structures apparently not directly involved in myogenesis.

MATERIALS AND METHODS

MATERIALS AND METHODS

Cells and Growth Conditions

L6 9/1 myoblasts were obtained from Dr. B.D. Sanwal (University of Western Ontario) and were a subclone of the original L6 myogenic cell line isolated by Yaffe (1968). These cells were maintained as subconfluent monolayers on the surface of glass or plastic culture dishes in α-minimal essential medium (MEM) (Stanners *et al.*, 1971) supplemented with antibiotics (100 units penicillin G per ml and 0.0685 mg/ml streptomycin sulphate) and either 10% (v/v) calf serum or 10% (v/v) fetal bovine serum as previously described (Wright, 1973). Glass plates were cleaned by soaking them overnight in a solution of concentrated acid (5% nitric in conc. sulfuric acid, v/v) followed by thorough rinsing in distilled water and pretreatment with $Mg(C_2H_2O_2)_2$ (Monahan, 1976). Fresh subclones were isolated by adding 100 cells to 100 mm culture plates containing growth medium and screening the resulting colonies for the ability to initiate fusion. Those judged to be fusion-competent were transferred, with the aid of sterile Pasteur pipettes and glass cloning discs, to 60 mm plates and were incubated at 37°C until a monolayer approached confluence. The cells were then removed with a trypsin (Difco) treatment of 0.2% trypsin in phosphate-buffered saline (PBS), pH 7.0 (containing in g/litre NaCl, 8.0; KCl,0.2 Na_2HPO_4, 1.15; KH_2PO_4, 0.2) and transferred to a 100 mm plate and again grown to near confluence. These cloned lines were then used immediately in a selection for con A-resistant myoblast lines.

To induce differentiation, cells were removed with 0.2% trypsin and 5 x 10^4 cells were added to 60 mm culture plates containing 4 ml of α-minimal essential medium with 10% calf serum. Culture medium was changed every two days until confluence with no additional changes thereafter. In some experiments, 2 ml of medium was exchanged every two days.

Cell fusion was quantitated by determining the proportion of nuclei within myotubes after the monolayers had been treated with dimethylsulfoxide: 10^{-3} M $ZnCl_2$ (1:4) for five minutes to slightly plasmolyse the cell (a condition under which the nuclei are easily distinquished). The cells were then fixed with methanol (50% for 30 seconds) and stained with methylene blue. At least 1000 nuclei were counted in randomly selected fields (10 to 15) at 200 x magnification.

All wild-type populations were discarded after 20 passages (1 x 10^5 cells cultured to 3 to 4 x 10^6 cells/100 mm plate) and fresh cultures were routinely obtained from cells frozen at early passages at -76° C in growth medium plus 5% dimethylsulfoxide.

Human skin fibroblast cultures were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital. Cell cultures from Duchenne dystrophic male patients were WG448 (6 years) WG840 (5.5 years), WG730 (15.5 years). Control cultures from normal

male children were MCH 57 (17 years), MCH 52 (7 years), MCH50 (6 years). Several fibroblast cultures were gifts from Dr. K. Wrogemann: GM323 and GM3781 were normal and dystrophic fibroblasts from normal normal and dystrophic 11 year old males respectively. These cultures were originally obtained from the Institute for Medical Research, Camden, N.J. A culture from a skin biopsy taken from a local Duchenne muscular dystrophy patient (12 years) was designated "Anderson".

The cells were grown in MEM supplemented with 10% foetal bovine serum as monolayers on 100 mm plastic culture dishes, or on 60 mm plastic culture dishes prior to ^{125}I con A binding experiments. Cells were removed from the dish surfaces by rinsing with a sterile phosphate buffered saline containing 0.1% trypsin (twice crystallized, from Sigma) and 0.02% EDTA. When the cells had completely rounded, they were aspirated gently into warm medium. A passage number was considered to be one doubling, and all experiments were performed before 20 passages in culture.

Con A Survival Curves and Variant Selection

The efficiency of colony formation in the presence of con A was measured by adding appropriate numbers of myoblasts, counted with a cell counter (Coulter Electronics Co) and not exceeding 1×10^5 cells/100 mm plate to 10 ml of α-minimal essential medium supplemented with 10% fetal bovine serum containing con A at various concentrations (Wright, 1973). Con A medium was prepared as a 60 µg/ml stock solution according to Wright (1973). After a suitable period of incubation at

37°C or 34°C the plates were drained and a saturated solution of methylene blue in 50% ethanol was added for about 10 min. The plates were then rinsed in water and the stained colonies on each plate were counted. Plating efficiencies were estimated by dividing the number of colonies counted by the number of cells plated. Relative plating efficiency was defined as the plating efficiency in the presence of the drug divided by the plating efficiency in the absence of drug. The D_{10} value is the concentration of drug which reduces the relative plating efficiency to 10%.

When the efficiency of colony formation in the presence of con A but in the absence of serum was measured, cells from a stock culture dish were treated with trypsin then seeded at various numbers in 100 mm plastic culture dishes containing 10 ml of α-MEM supplemented with 10% (v/v) foetal bovine serum. After 36 hours of incubation at 37°C, the medium was aspirated and the plates were washed twice with 5 ml of PBS at 37°C. α-MEM containing con A was added and the plates were incubated at 37°C for 36 hours. At that time, the plates were washed with the following sequence of solutions: a) once with PBS at 37°C; b) once with warm PBS containing 0.1 M α methyl glucoside; c) once again with warm PBS. The dishes were finally flooded with α-MEM supplemented with 10% foetal bovine serum then incubated at 37°C for 10 days at which time sizable colonies had formed.

Con A-resistant variants were selected in a single step from independent clones at a concentration of con A (50 to 55 μg/ml) which would permit the survival of less than 1×10^{-5} cells. Freshly cloned

myoblast populations (see "Cells and Growth Conditions" above) were added at a density of 1 x 10^5 cells/100 mm plates (a total of 25 plates/experiment were used) and incubated at 34°C or 37°C for 3 weeks. Plates were then examined for colonies containing more than 20 cells, and these were transferred, using sterile Pasteur pipettes and glass cloning discs, to 60 mm culture plates containing growth medium supplemented with 10% foetal bovine serum. When the cultures reached partial confluence, stock cultures were made without further exposure to con A while the remaining portion of each population was tested (as described above) for their ability to form colonies in 50 µg/ml con A. Those clones capable of a high relative efficiency of colony formation were chosen for further study. It should be noted that the selection procedure involves a single exposure to the lectin.

Creatine Phosphokinase Activity

The activity of creatine phosphokinase (CPK) in differentiating myoblasts was determined by the method of Shainberg *et al.*, (1971) using 10 µM diadenosine phosphate to inhibit adenylate kinase (Leinhard and Secemski, 1973). Protein was measured by the method of Lowry *et al.*, (1951).

Mannose Transferase Activity

Incorporation of [^{14}C]-mannose from GDP-[^{14}C]-mannose (Amersham Corp.) into endogenous lipid and oligosaccharide-lipid was measured by a modification of the procedure described by Krag and Robbins (1977). The 80 µl incubation mixture contained 20 mM Tris, pH 7.4,

0.15 M NaCl, 0.2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 10 µM UDP-N-acetylglucosamine, 15 µM GDP-[^{14}C] mannose (100 nCi), 4 mM AMP to reduce pyrophosphatase activity (Vessey and Zakim, 1975), and various amounts of a crude membrane fraction freshly prepared from subconfluent myoblasts grown on the surface of 100 mm plastic tissue culture plates (3.5 to 4.3 x 10^6 cells/plate). After 2 to 15 minutes at 34°C the reaction was stopped by the addition of 3 ml of chloroform: methanol (2:1) at room temperature.

Glycolipid soluble in chloroform:methanol (2:1) was isolated by two additional 15 minute extractions each followed by centrifugation for 10 minutes at 1500 x g. Nonlipid radioactivity was removed from the combined extracts by washing three times with 2 ml of 0.9% saline: methanol (2:1).

The pellet was dried under N_2 and resuspended, by sonication in 2 ml of water, centrifuged, and followed by repeated water:methanol (1:1) washes, until radioactivity in the supernatant approached background levels. Oligosaccharide-lipid soluble in chloroform: methanol:water (10:10:3) was obtained by two 15-minute extractions of the washed pellet.

Chloroform and methanol were removed from all fractions by evaporation under N_2. Radioactivity was determined by liquid scintillation counting after samples were dissolved in 10 ml of ACS (Amersham Corp.). The addition of dolichol-PO_4 (Sigma Chemical Co.) to the assay mixture was performed in tubes containing various amounts of the lipid solubilized in chloroform:methanol (2:1) and dried under a stream of N_2. Twenty µl of 0.8% Triton X-100 in reaction buffer without saline was

added. Samples of membrane protein were added to the tubes and the reaction was started by the addition of 40 µl of assay buffer without saline containing 25 nCi of GDP-^{14}C-mannose. The reaction was terminated after 15 min at 37°C by the addition of 3 ml of chloroform: methanol (2:1).

Attempts were also made to measure mannosyl transferase activity in permeabilized whole cells. Permeabilization was carried out according to the method of Billen and Olson (1978). Cells were harvested after treatment with trypsin by washing each 100 mm culture dish with 3 ml of PBS followed by centrifugation. The cell pellet was washed in an equal volume of 0.25 M sucrose in distilled water and again collected by centrifugation. The cell pellet was resuspended in a small volume of 0.25 M sucrose, 1% Tween 80 and incubated for 90 minutes at room temperature. A volume of cell suspension containing 10^7 cells was distributed to separate test tubes and the cells harvested by centrifugation at 500 x gravity. The cell pellet was resuspended in 0.25 ml of α-MEM followed by addition of 40 µl of the assay mixture containing GDP[^{14}C]-mannose (100 nCi). The reaction was stopped by adding 3 ml of chloroform:methanol (2:1).

Metabolic Labelling of Glycoproteins

The medium from logarithmically growing, subconfluent myoblasts was removed and the cells were washed once with sterile PBS. Fresh growth medium (4 ml) supplemented with 2% calf serum was then added to the 100 mm plastic tissue culture plates. Two µCi of [^{14}C]-mannose was added and incubation was continued at 37°C for 8 hours

longer. At that time, medium was removed, the monolayer was layered with 1 ml of water and the cells were scraped from the surface with a rubber policeman. Two ml of chloroform:methanol (2:1) were added to the cell suspension in a test tube and the mixture was thoroughly vortexed and then centrifuged. The bottom layer was removed and saved while the interface and upper aqueous layer were re-extracted with another 1 ml of chloroform. The aqueous phase and interface which remained after this extraction were used to isolate membrane glycoproteins. One ml of methanol was added to solubilize any remaining chloroform and the insoluble material was isolated by centrifugation. Washes and oligosaccharide-lipid extractions were continued as described above, leaving the final glycoprotein preparation in a dried pellet. This was stored at -20°C until used in the analysis of labelled sugars.

Labelling the carbohydrate portion of glycoproteins with [^{3}H]-mannose before cytoplasmic membrane isolation was accomplished by removing the growth medium of exponentially growing cells, and replacing it with fresh medium containing 2 μCi/ml of 2-[^{3}H] mannose (New England Nuclear, 17.3 Ci/mmole). The cells were incubated for one generation in labelled medium.

Identification of Labelled Sugars in Glycoprotein Fraction

After extraction of lipid and oligosaccharide-lipids as described above, glycoprotein fractions (1.5 to 2.0 mg per 100 mm plate, 5 to 8 x 10^3 dpm) were hydrolysed with 0.3 N HCl at 100°C for 16 hours in a sealed tube under N_2. Radiolabelled monosaccharides were developed on a paper chromatographic system we have previously described which is

capable of separating mannose and fucose (Wright *et al.*, 1979). Quantitative determination of the radioactivity was obtained by elution from the chromatograms, of areas known to contain peaks, directly into liquid scintillation vials followed by freeze-drying and scintillation counting (Wright *et al.*, 1979). After elution of radioactive strips the paper was dried and counted to ensure that complete elution of radioactivity had occurred.

[^{3}H]-labelled Con A Binding to Cell Surfaces

Wild type and con A-resistant cells were removed with trypsin solution (0.2% Bacto trypsin in phosphate buffered saline, pH 7.0) from culture dishes and resuspended to 2.5 x 10^4 cells/ml in MEM containing either 10% calf serum or 10% fetal bovine serum (Gibco). Four ml of the cell suspension were added to 60 mm plastic culture dishes (Lux Scientific Co.) and incubated at 37°C for about 72 h or until the monolayer began to approach confluence (1.1 to 1.6 x 10^6 cells/dish). The dishes were cooled on ice for 5 min followed by two washes of 2 ml each with ice-cold 0.85% saline containing 10 mM NaN_3, pH 7.0. The monolayers were covered with an additional 2 ml of cold saline and left on ice for 40 min. The saline was removed and binding was started by flooding the dish with 1 ml of ice-cold saline supplemented with various concentrations of con A (Calbiochem) prepared before each experiment from the lyophilized powder. [^{3}H] acetyl-N-acetylated con A (25.2 Ci/mmole) was added to a final activity of 0.5 µCi/µg or 22 µCi/µg. The dishes were incubated on ice for a further 20 min at which time maximum binding was attained. The dishes were

agitated periodically to ensure complete coverage of the monolayer by the solution. The lectin binding solution was then removed and the cells were washed 5 times with 2 ml of the ice-cold saline. Monolayers were solubilized in 1 ml of 10% triton X-100 overnight at 37°C. The dishes were scraped with a rubber policeman to remove remaining cellular fragments and the entire triton X-100 solution was transferred to a scintillation vial to which was added 10 ml of ACS (Amersham) cocktail. Radioactivity was determined in a liquid scintillation counter. In some experiments con A binding was performed at 37°C in the absence of NaN_3. In this case cells were washed with warm PBS (37°C) and incubated at 37°C in the presence of saline supplemented with various concentrations of labelled con A. There was specificity in the binding of labelled lectin since the amount of binding at all concentrations tested in the presence of 0.2 M methyl α-D-mannoside was 3 to 5% of the binding which usually occurred in the absence of inhibitor. The quantity of binding in the presence of the sugar was subtracted from the binding data obtained in the absence of the hapten (Parfett *et al.*, 1981; Wright and Ceri, 1977b).

Preparation of Cell Surface Membrane

Twenty 100 mm plastic culture dishes (Lux Scientific Co.) containing 10 ml of MEM supplemented with 5% fetal bovine serum, were seeded with 10^5 cells, and incubated at 37°C until the monolayer approached confluence. The medium was aspirated and the cells rinsed once with 5 ml of PBS. Five ml of ethylene diamine tetraacetic acid (EDTA) solution in PBS (0.2 g/l) was added and the cells were scraped from

dishes with a rubber policeman when they appeared fully rounded. Cells were harvested by centrifugation and washed once in 40 ml of PBS. Cell surface membranes were obtained after disruption by 10 to 20 manual strokes of a tight fitting Dounce homogenizer by the method of Brunette and Till (1971), except the polyethene-dextran two phase system was centrifuged only once at 100 x g for 10 min to ensure adequate amounts of protein were obtained for analysis by SDS gel electrophoresis.

SDS Slab Gel Electrophoresis

Slab gels (0.2 cm x 25 cm x 11.5 cm) were prepared and used according to the method of Laemmli (1970). For each track, 50 to 100 µg purified membrane protein in 10 µl of sample buffer was solubilized by heating to 100°C for 3 to 5 min. Electrophoresis was carried out at a constant current of 25 m Amperes, until the bromophenol blue tracking dye had reached the bottom of the separating gel (6 to 7 h). Proteins were fixed overnight in water:methanol:acetic acid (46:46:8). The gels were either dried onto Whatman #1 filter paper or stained with Coomassie brilliant blue R and destained before drying. The slab gel was calibrated using proteins of known molecular weight.

The distribution of ^{3}H-mannose labelled glycoproteins within each gel track was determined by simultaneously cutting 1.5 mm slices of the dried gel tracks to be compared, and estimating the radioactivity in each slice according to the method of Ames (1974).

Binding of [^{125}I]-Con A to SDS-PAGE Gels

Con A was iodinated with [^{125}I] by a modification (Tanner and

Anstee, 1976) of the method described by Hunter and Greenwood (1962) using affinity chromatography on Sephadex G-75 to re-purify the labelled lectin. Gels were fixed then equilibrated in Tris-buffered saline containing 0.01% NaN_3 before staining with [^{125}I] -con A (0.5 μCi/ml) according to the method of Horst *et al.*, (1980). Con A labelled areas within the dried gel were visualized by autoradiography using Kodak X OMAT RP-1 film for up to seven days at room temperature.

Affinity Chromatography on Con A-Sepharose

Protein bands containing ^{3}H-mannose labelled glycoproteins were sliced from the dried, stained slab gels and rehydrated in 1 ml of 0.1 M Tris-HCl, pH 8.0 containing 1 mM $CaCl_2$ and 2 mg/ml of pronase (Sigma Chemical Co.). The gel slices were crushed in a 2 ml glass tissue homogenizer (Belco Biological Glassware) and incubated for 48 h at 37°C under a toluene atmosphere. An additional 2 mg of pronase was added after 24 h of incubation. Boiling (2 min) terminated digestion.

Gel fragments were removed by centrifugation (100 x g for 10 min). The supernatant containing the released glycopeptides was loaded onto a 5 cm x 0.6 cm con A sepharose column (Sigma Chemical Co.) previously equilibrated with 0.01 M Tris HCl, pH 7.5 containing 0.001 M $CaCl_2$, $MnCl_2$, $MgCl_2$; 0.02% NaN_3; 0.1 N NaCl. The columns were washed with 10 ml of the buffer, followed by elution of bound components with a linear gradient of α methyl-D-mannoside where 0.2 M was the limit concentration. Twenty drop fractions were collected and analysed by scintillation counting.

3-hydroxy-3-methyl-CoA Reductase Assay

(Mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC1.1.1.34

The assay for this enzyme performed essentially as described by Shapiro *et al.*, 1974.

Cells grown on 100 mm tissue culture dishes were harvested after EDTA treatment as previously described and washed twice in 10 ml of PBS, suspended in PBS and ruptured with ultra sonic energy. After centrifugation for 10 min at 12,000 x g, the supernatant with suspended microsomal membranes was centrifuged for 1 h at 48,000 x g and the pellet resuspended for assay in a small volume of buffer by gentle homogenization. 100 to 400 µg of microsomal protein were mixed with sufficient buffer (30 mM EDTA, 250 mM NaCl, 1.0 mM dithiothreitol, 50 mM potassium phosphate, pH 7.4) to bring the volume to 100 µl. After a 5 min preincubation at 37°C 50 µl of cofactor substrate solution (4.5 µmoles of glucose-6-phosphate, 0.3 I.U. of glucose 6-phosphate dehydrogenase, 450 nmoles of $NADP^+$ and 50 nmoles of DL-hydroxymethyl-[3-^{14}C]glutaryl CoA (specific activity 7500 dpm/nmoles) and 30,000 dpm/100 µl of [5-^{3}H] mevalonic acid as an internal standard. The reactions were stopped after 15 min by the addition of 25 µl of 10 M HCl and incubated a further 30 min at 37°C to permit the mevalonic acid to lactonize. Protein was removed by centrifugation and the mevalonic acid in 100 µl of supernatant was recovered by this layer chromatography in benzene-acetone. The amount of [^{14}C] mevalonic acid synthesized was detected by scintillation counting and corrected for recovery by use of the [^{3}H] mevalonic acid internal standard.

Membrane Transport of [^{3}H]-Mannose

To investigate the relative rates of uptake in wild-type and con A-resistant myoblasts, 10^5 cells were seeded in 35 mm plastic culture dishes in 2 ml of α MEM supplemented with 10% calf serum. To ensure an even distribution of cells in the small plates, they were not disturbed for 20 minutes after addition, to allow the cells to settle and firmly attach to the dish surface. Dishes were incubated at 37°C for 48 h at which time, cells were subconfluent. The medium was aspirated and 0.5 ml of fresh medium supplemented with 0.1% calf serum, 0.5 μCi of [^{3}H]-mannose and 0.2 mM cold mannose was added. Incubation for various times was continued at 37°C in a 5% CO_2 atmosphere. The medium was quickly aspirated and the plate was washed 5 times with 2 ml of ice-cold PBS. The cell monolayer was solubilized in 1 ml of 10% Triton X-100 before scintillation counting in 10 ml of ACS (Amersham). This method is similar to the one reported by (Li and Kornfeld, 1977).

Assay of Plasminogen Activator by Casein Hydrolysis

The presence of plasminogen activator was detected by proteolysis of casein essentially as described by Goldberg (1974). Five 100 mm culture dishes were seeded with 200 cells of each line and were incubated at 37°C until colonies of 100 to 200 cells had formed. The dishes were rinsed twice with 5 ml of PBS at 37°C and colonies on the surface were overlayered with 10 ml of α MEM containing 0.4% agarose, 1.5% casein and 10% (v/v) human serum. Incubation was continued for 15 h at which time the plates were observed, followed by 24 additional hours. Dishes were then photographed and areas of casein hydrolysis were

scored. The agarose overlay was removed under water, followed by the staining of colonies in the usual manner.

Quantitation of Chromosomes

5×10^5 cells of each line to be investigated were seeded in a 100 mm plastic culture dish containing 10 ml of medium. Incubation at 37°C was continued for 24 h before colchicine was added to a final concentration of 10 µg/ml from a sterile stock solution of 250 µg/ml in α MEM. After 2 to 3 h of incubation at 37°C, the medium was gently removed from the monlayer by aspiration and the dishes were flooded for 1.5 min with 5 ml of 0.2% Bacto Trypsin solution. The solution was swirled several times to dislodge the rounded cells while leaving attached most non-mitotic cells.

Harvested mitotic cells from a single dish were collected by centrifugation and resuspended in a drop of 75 mM KCl at 37°C, then diluted to 5 ml in the same solution. The cells were allowed to swell for 20 to 30 minutes and again harvested by centrifugation (240 x g). All but 0.5 ml of the hypotonic KCl was aspirated and the swollen cells were again gently resuspended.

Fresh, ice-cold methanol-acetic acid (3:1) was added slowly by running small drops down the inside of the centrifuge tube and mixing the suspension of cells simultaneously until about 3 ml of the fixative had been added (10-12 minutes). Cells were held in this mixture for a further 15 min on ice before being harvested by centrifugation. Two additional cycles of fixation were performed, but the fixative was added more quickly. After the final centrifugation, the cells were

resuspended in a small volume of fixative and dropped from 50 cm onto a cold, wet glass microscope slide, then air dried.

Metaphase spreads were observed on a Ziess Optivar photomicroscope at a magnification of 400 x using phase contrast optics. Chromosomes were counted on photographs of from 50 to 60 spreads for each line (final magnification 1880 x).

RESULTS

RESULTS

Section I Isolation of Con A-resistant Myoblasts From Wild Type Populations

A Selection of Independent Wild Type Myogenic Populations

In order to establish a relationship between changes in cellular structures and altered cellular function, it is necessary to attempt several independent experiments. Only in this fashion will it be apparent that two cellular properties vary independently, or alternatively, that they change in a manner which would suggest a biochemical lesion in the selected cell leads directly to altered cell surface structures and a new behavioural phenotype. L6 myogenic lines present an added complication to analysis of structure-function relationships which are components of the developmental process. During routine culture of a wild type population, nondifferentiating variants begin to accumulate in the population to such an extent that after 50 to 60 passages, less than 20% of the population may be able to undergo the myogenic process (Yaffe, 1973). Consequently, a wild type population must be carefully examined for the frequency of monmyogenic variants before mutant selections are begun.

To ensure that nonfusing variants were not present in significant numbers at the beginning of a con A selection, wild type populations were freshly cloned, and clones chosen which displayed superior abilities to initiate and undergo myogenesis. Figure 5 illustrates

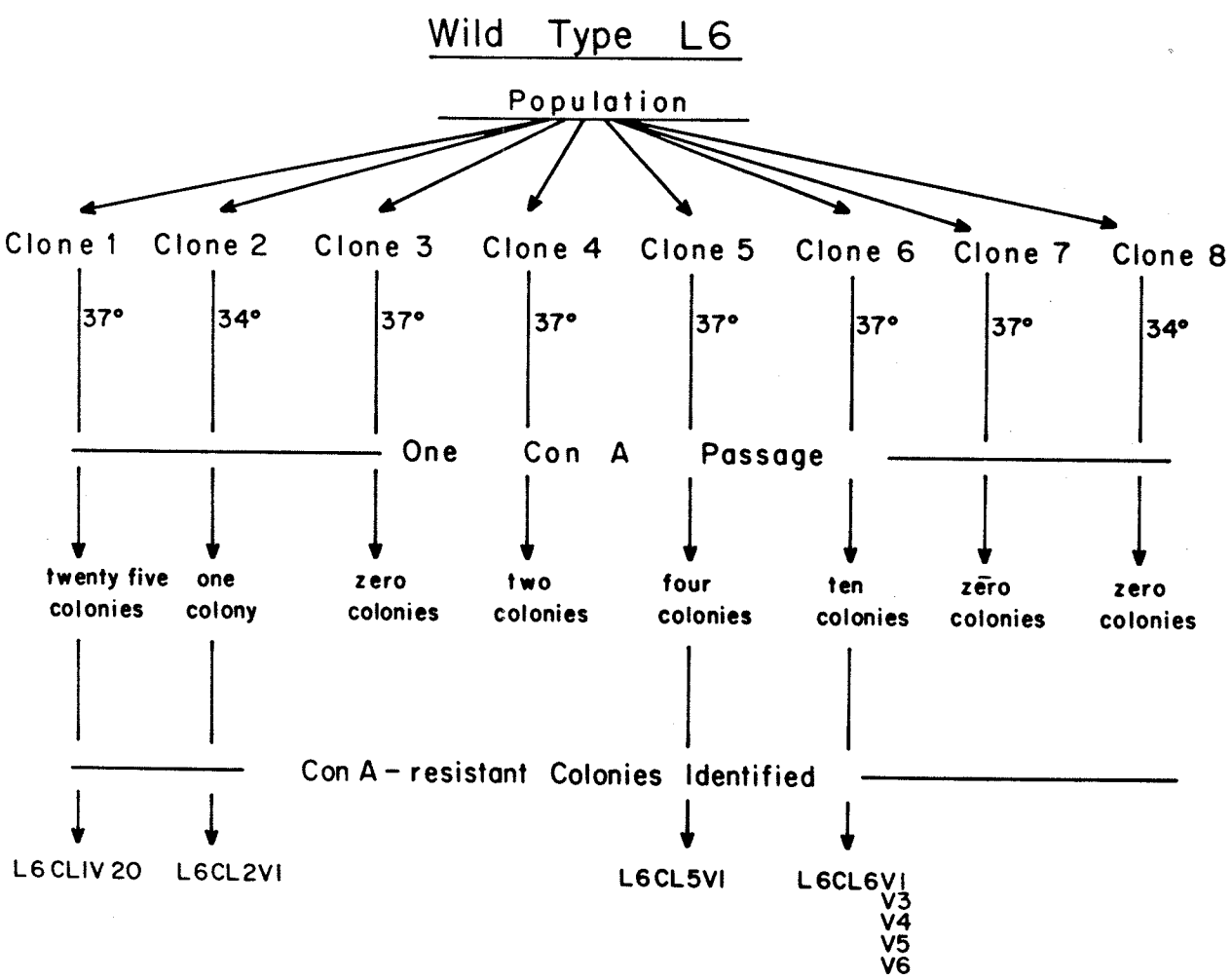
Figure 5: Photomicrographs of myoblast colonies which have initiated membrane fusion. X40. Cells were stained with methylene blue in H_2O: CH_3CH_2OH (1:1).

the appearance of several fusion-competent colonies after about 10 days of growth in αMEM supplemented with 10% foetal bovine serum. At the colony centre myogenesis has begun, resulting in areas with fused cytoplasmic membranes and condensation of nuclei into limited areas within the growing syncytium. This characteristic feature can be visualized in living cultures by either phase contrast or normal light microscopy at low power. If the colony appeared to have arisen from a single cell, it was isolated within a cloning cylinder and the cells were removed after trypsin treatment (see Materials and Methods). Since myogenesis results in terminal differentiation, only the population of single cells which are near the outer edge of the colony, and not yet committed to myogenesis can continue to grow. Consequently, wild type populations of myogenic cells used in the selections reported in this study were derived from cells at the outer edges of fusion-competant colonies.

B Selection of Con A-resistant Myoblasts

Figure 6 summarizes the selection scheme used to isolate con A-resistant L6 myoblasts from independent fusion-competant wild type clones. The important feature in this scheme is that non-mutagenized cells were exposed to only a single challenge with the cytotoxic lectin. Moreover, the procedure minimized the number of passages required between the initial isolation of each wild type clone and the identification of a con A-resistant variant derived from it. Although eight independent selection experiments were performed according to

Figure 6: Summary of the scheme used to isolate independent con A-resistant myoblast lines.

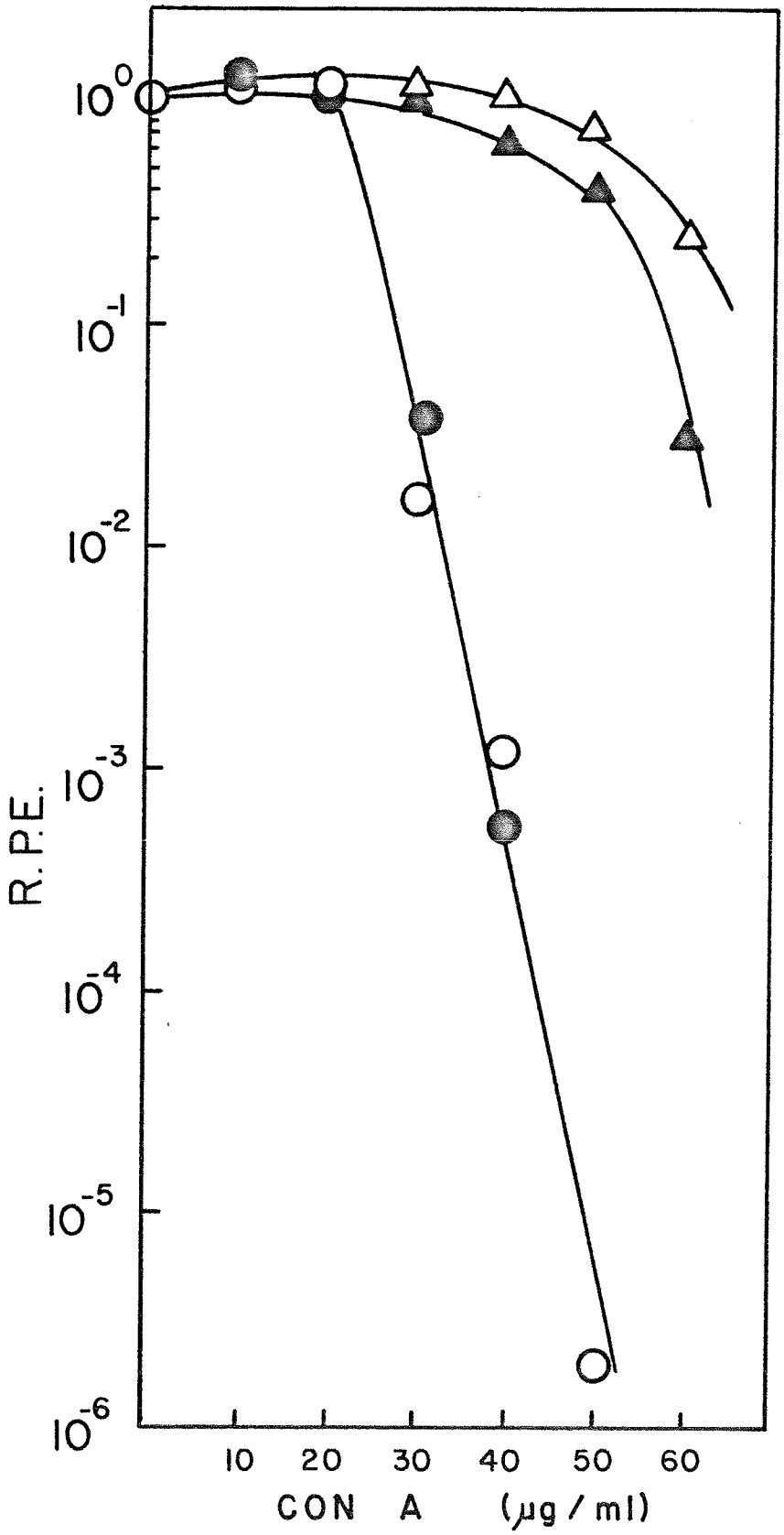

Wild Type L6
Population
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
37°
34°
37°
37°
37°
37°
37°
34°
One Con A Passage
twenty five colonies
one colony
zero colonies
two colonies
four colonies
ten colonies
zero colonies
zero colonies
Con A - resistant Colonies Identified
L6CLIV 20
L6CL2VI
L6CL5VI
L6CL6VI
V3
V4
V5
V6

this procedure, only four attempts gave rise to surviving colonies containing greater than 20 cells. From a total of 40 surviving colonies in these four productive selections, only eight were found to produce populations of cells resistant to con A after growth in medium not containing the lectin, as judged by colony forming ability in 50 µg/ml con A. Since each of the eight selection experiments began with the plating of 2.5×10^6 cells, it was possible to estimate that the isolation frequency for con A-resistant L6 myoblasts in the presence of 50 µg/ml of lectin was 1 in 2.5×10^6 cells.

C. Sensitivity of Wild Type and Con A-resistant Myoblasts to Con A Cytotoxicity

Figure 7 shows the ability of L6CL2V1 and L6CL5V1, two independent myoblast lines, to grow and form colonies in the presence of various con A concentrations. The range of lectin concentrations was limited to 0 to 60 µg/ml con A since preparation of medium supplemented with con A results in precipitation of some serum components. Wright (1973) has shown that about 5% of the con A is lost if concentrations of less than 60 µg/ml are used. Both cell lines are clearly more resistant to the cytotoxic effects of the lectin than their parental wild type lines. For example, the wild type lines exhibited a 50% reduction in colony forming ability (D_{50}) at about 23 µg/ml of con A, whereas the D_{50} values for L6CL2V1 and L6CL5V1 were determined to be 45 and 52 µg/ml lectin respectively. This is an approximately two-fold increase in D_{50} estimate for the myoblast variants and is similar to the results obtained with con A-resistant Chinese hamster ovary cell lines isolated

Figure 7: Effects of various concentrations of con A on the colony forming ability of wild type and con A-resistant myoblast lines. ●, wild type clone 2; ▲, L6CL2V1: o, wild type clone 5; and Δ, L6CL5V1.


R.P.E.
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10
20
30
40
50
60
CON A (μg / ml)

in this and other laboratories (Ceri and Wright, 1978a; Krag, 1979; Briles *et al.*, 1978).

The remaining six variant myoblast lines selected from wild type 1 and 6 (Figure 6) were tested for lectin sensitivity by determining relative colony forming ability in the presence of 50 µg/ml con A, the lectin concentration used in the selection experiments (Table I). It is obvious that these cell lines are also significantly more resistant to con A than the parental wild type lines from which they were obtained. In the presence of 50 µg/ml lectin, all but one variant line was slightly reduced in plating efficiency (20 to 90% reduced) but still exhibited relative colony forming abilities that were 10^3 to 10^5 times higher than their parental cells. For comparison, single point tests of L6CL2V1 and L6CL5V1 were included in Table I. Since all the clones tested in this fashion compare well with the relative plating efficiencies at 50 µg/ml con A shown in Figure 7, it was concluded that the resistance properties of variants selected from wild type clones 1 and 6 and variants selected from wild type clones 2 and 5 are very similar. The increase in D_{50} is likely to be approximately two-fold. An example of the type of data obtained in experiments performed to determine the relative plating efficiency of L6CL2V1 and its parental wild type in 50 µg/ml con A is illustrated in the photograph shown in Figure 8.

Killing curves of this type are subject to some inherent inaccuracy due to the effects of increasing cell number on the relative plating efficiency. Figure 9 shows that in the presence of 30 µg/ml con A, the relative plating efficiency drops by 40% when the number of

Figure 8: Photograph of colony formation by wild type clone 2 and L6CL2V1 myoblasts in normal medium or in medium with 50 µg/ml con A.

wild type clone 2	upper left: 200 cells, no con A upper right: 10^5 cells, 50 µg/ml con A
L6CL2VI	lower left: 200 cells, no con A lower right: 1000 cells, 50 µg/ml con A.

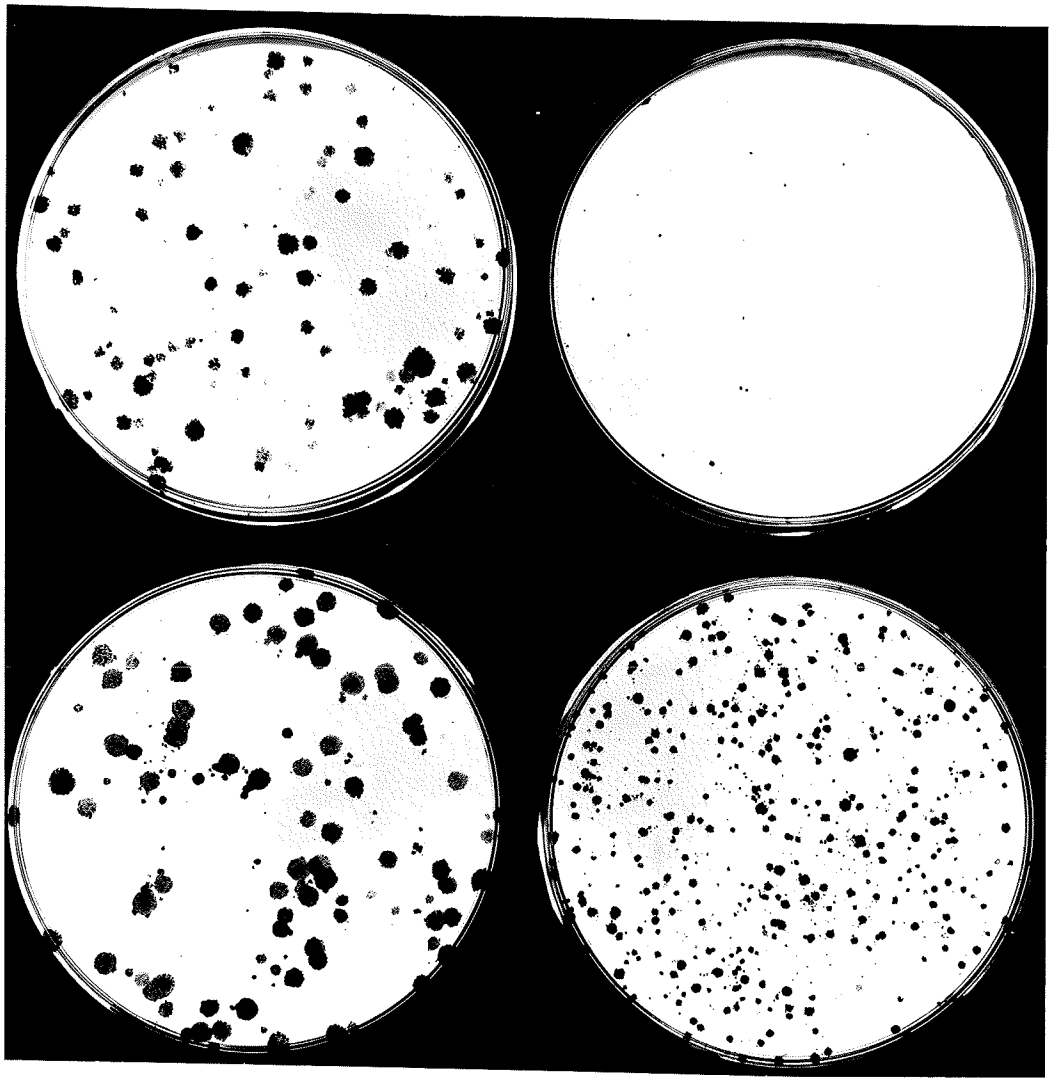
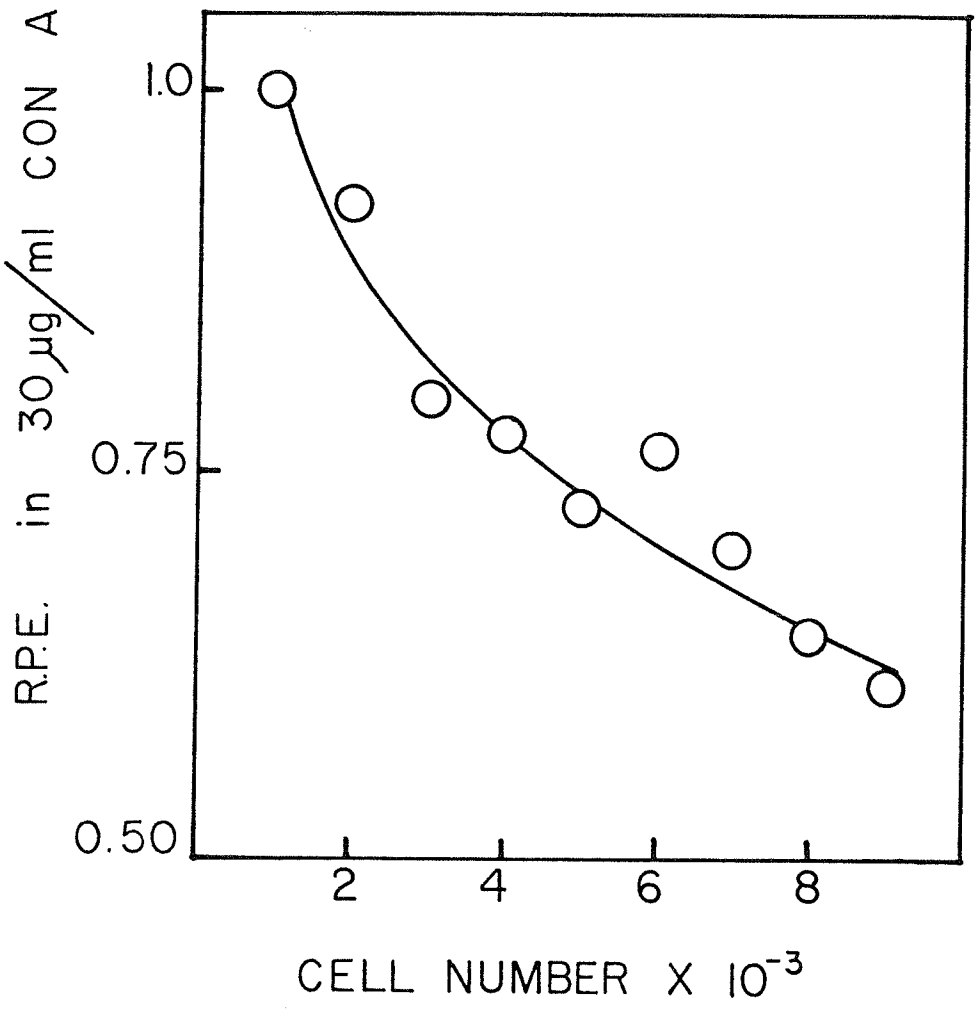


Figure 9: The effect of increasing cell number on the relative plating efficiency of L6CL2V1 myoblasts. Increasing numbers of the con A-resistant line were plated in 30 µg/ml con A. Colonies greater than 20 cells were counted after 10 days of growth at 37°C. Plating efficiency is expressed relative to the number of colonies which formed on the plate inoculated with 1000 cells.


R.P.E. in 30 µg/ml CON A
1.0
0.75
0.50
2
4
6
8
CELL NUMBER X 10^{-3}

myoblasts on a culture dish is increased from 1000 to 9000. Reduced colony formation in the presence of the drug hydroxyurea and at high cell densities has been observed previously in this laboratory (Lewis and Wright, 1978). For this reason, several concentrations of cells were used at each con A concentration and the highest relative plating efficiency was plotted (Figure 7) in an attempt to minimize reductions in plating efficiency at high cell numbers.

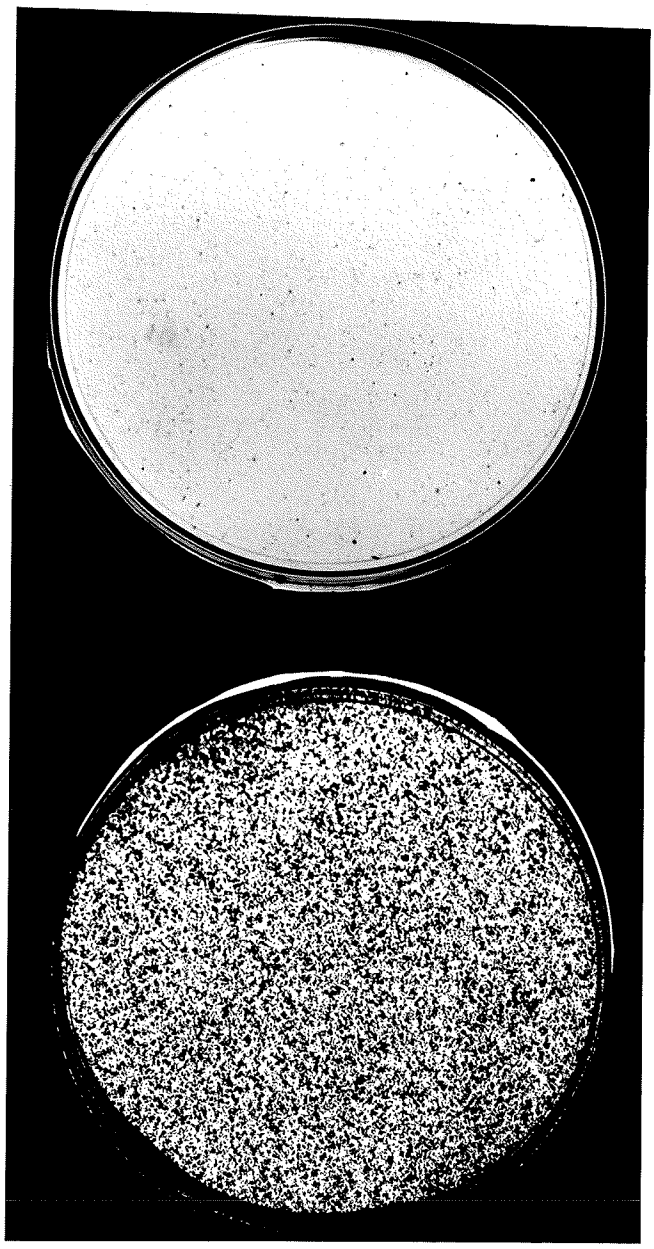
Stability of con A-resistance during extended culture: to determine if extended growth of variant myoblasts in the absence of the lectin resulted in any decrease in plating efficiency or growth in the presence of the drug, resistant cells which had been in culture for more than six months were plated at high cell number (100,000 cells per dish) in the presence of 60 µg/ml con A. This concentration of lectin is more than that required to kill all of the wild type cells on the plate (see Figure 7); however, the variant cells quickly grew to confluence under the same conditions (Figure 10). This experiment was performed near the end of the study, after the cells had remained stored at -70°C for more than three years, and then cultured in the absence of a selective agent for six months. The con A-resistant phenotype must therefore be described as a stable alteration in the expression of cellular sensitivity to this lectin.

Cytotoxicity of con A in the absence of serum: to test the possibility that interaction between lectin and serum components may be a possible cause of the differential killing effect of con A for variant and wild type cells, the cytotoxicity of con A was examined

Figure 10: A comparison of myoblast growth in con A after 6 months of continuous culture in the absence of lectin. 10^5 cells were plated in 60 mm culture dishes containing 4 ml of medium supplemented with 60 µg/ml con A. Cells were stained with methylene blue in H_2O:CH_3CH_2OH (1:1) after 5 day's growth at 37^oC.

upper: clone 2 wild type

lower: $L6C_L2V1$



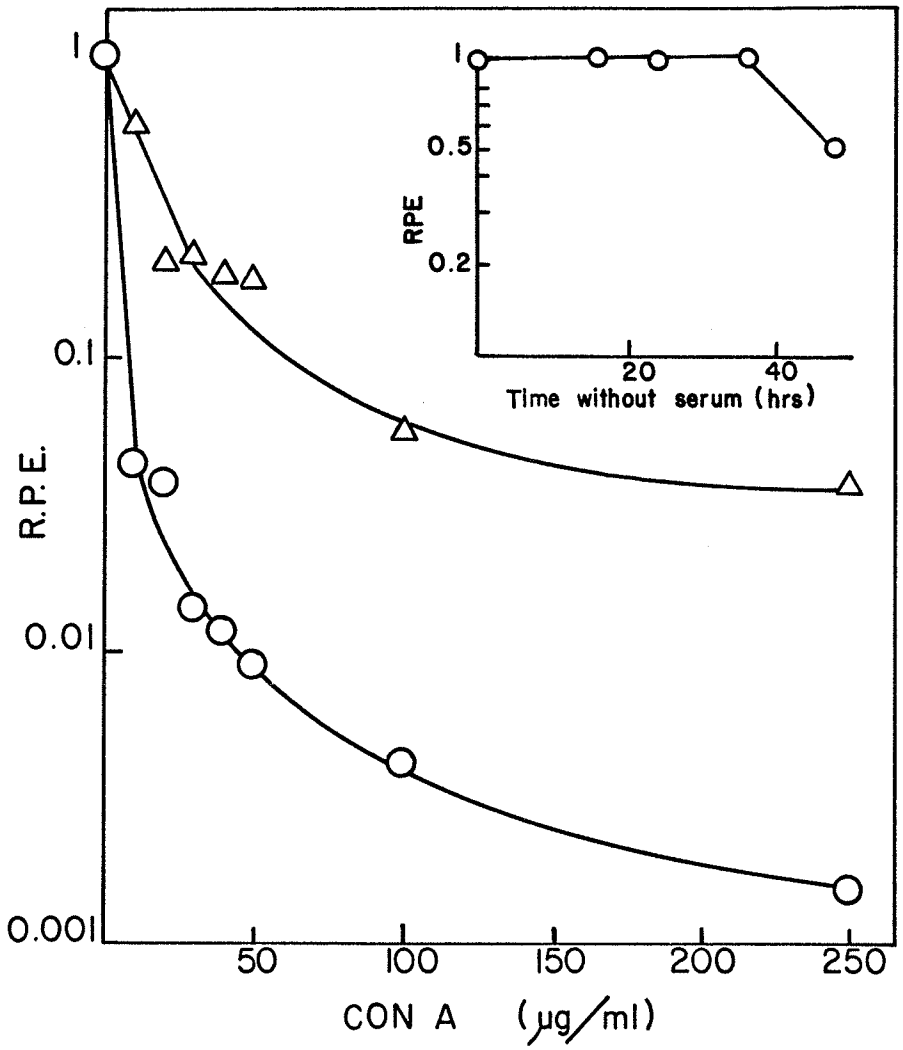
by a second type of killing curve in the absence of serum supplemented medium (Ozanne and Lurye, 1974). Serum complement, for example, is known to play an inhibitory role in lymphocyte activation after con A induced capping of the cytoplasmic membrane (Forsdyke, 1977).

Wild type clone 2 and L6CL2V1 cells were plated in growth medium for 36 hours. The medium was then replaced with αMEM not supplemented with serum, but containing appropriate concentrations of con A, as described in Materials and Methods. Higher concentrations of the lectin were required to cause a significant reduction in colony forming ability of both wild type and variant cells (Figure 11). This was probably due to the short time which cells were exposed to the lectin (36 hours). Times beyond 36 hours were not investigated because colony forming ability began to decrease in the absence of serum. The L6CL2V1 population was found to be less sensitive to con A than wild type clone 2 cells when assayed in this manner. Wild type clone 2 cells formed colonies with a relative efficiency of about 0.4% after exposure to 100 μg/ml con A, whereas the plating efficiency of L6CL2V1 was about 6% - more than a 10-fold difference. This experiment suggests that serum components are not required for the cytotoxic action of the lectin, a conclusion reached previously in this laboratory with Chinese hamster ovary lines selected for resistance to con A (Ceri and Wright, 1977). Furthermore, the serum components removed by precipitation with the lectin are unlikely to play a role in cytotoxicity. This idea is supported by the finding of Florini and Roberts (1979) that only two macromolecules (fetuin and insulin) are required for L6 myoblast growth. The slight amount of precipitate that usually developed

Figure 11: Effects of various concentrations of con A on the colony forming ability of wild type and con A-resistant myoblasts in the absence of serum. Myoblasts of each type were plated in normal medium for 35 hours prior to replacement by αMEM without serum and containing con A at increasing concentrations for an additional 36 hours. The medium containing lectin was then replaced with regular serum containing medium until colonies had formed. Inset: Effect of various times in medium without serum on colony-forming ability of wild type clone 2 cells.

o - o wild type clone 2

Δ - Δ L6CL2V1


1
0.1
0.01
0.001
R.P.E.
50
100
150
200
250
CON A (μg/ml)
1
0.5
0.2
RPE
20
40
Time without serum (hrs)

during medium preparation in this study would not be expected to remove significant quantities of either protein, since fetuin contains triantennary, complex oligosaccharides (Baenziger and Fiete, 1979a) and the insulin molecule is not a glycopeptide.

Section II Myogenic Development in Wild Type and Con A-resistant Myoblasts

A Morphological Differentiation of Wild Type and Con A-resistant Myoblasts

The most striking feature displayed by developmentally competent myoblasts is their morphological transformation from mononucleated single cells to multinucleated syncytia. The onset of cytoplasmic membrane fusion in myoblast cultures can be estimated by observing cultures by phase-contrast microscopy and determining the time that progressively growing multinucleated myotubes appear in fields selected at random (Yaffe, 1969). Under the culture conditions employed during this study, a burst of wild type myoblast fusion occurs one day after the cells have reached confluence on a 60 mm culture dish (Figure 12). A high degree of membrane fusion results in the formation of a branching, syncytial membrane network over large areas of the dish surface. Groups of condensed nuclei are also apparent within the common cytoplasm (Figure 13). In contrast, the cells of variant myoblast lines do not fuse to form multinucleated myotubes, nor do they align their long axes as do fusion-competant myoblasts

Figure 12: Fusion after growing myoblasts reach confluence. (a) Fusion index of wild type clone 2, (o) and the con A-resistant variant L6CL2V1 selected from it, Δ. (b) Fusion index of wild type clone 5, (o) and the variant L6CL5V1 selected from it. The fusion index was calculated as the percentage of nuclei located in fused elements containing at least three nuclei. One half of the medium was exchanged every 48 hours in the wild type culture dishes, but to ensure that the con A-resistant lines remained attached to the dish surface for the duration of the experiment, fresh growth medium was added every 24 hours. Arrows indicate loss of viability of the variant cell monolayers if medium is not changed every 24 hours (see Figure 20).

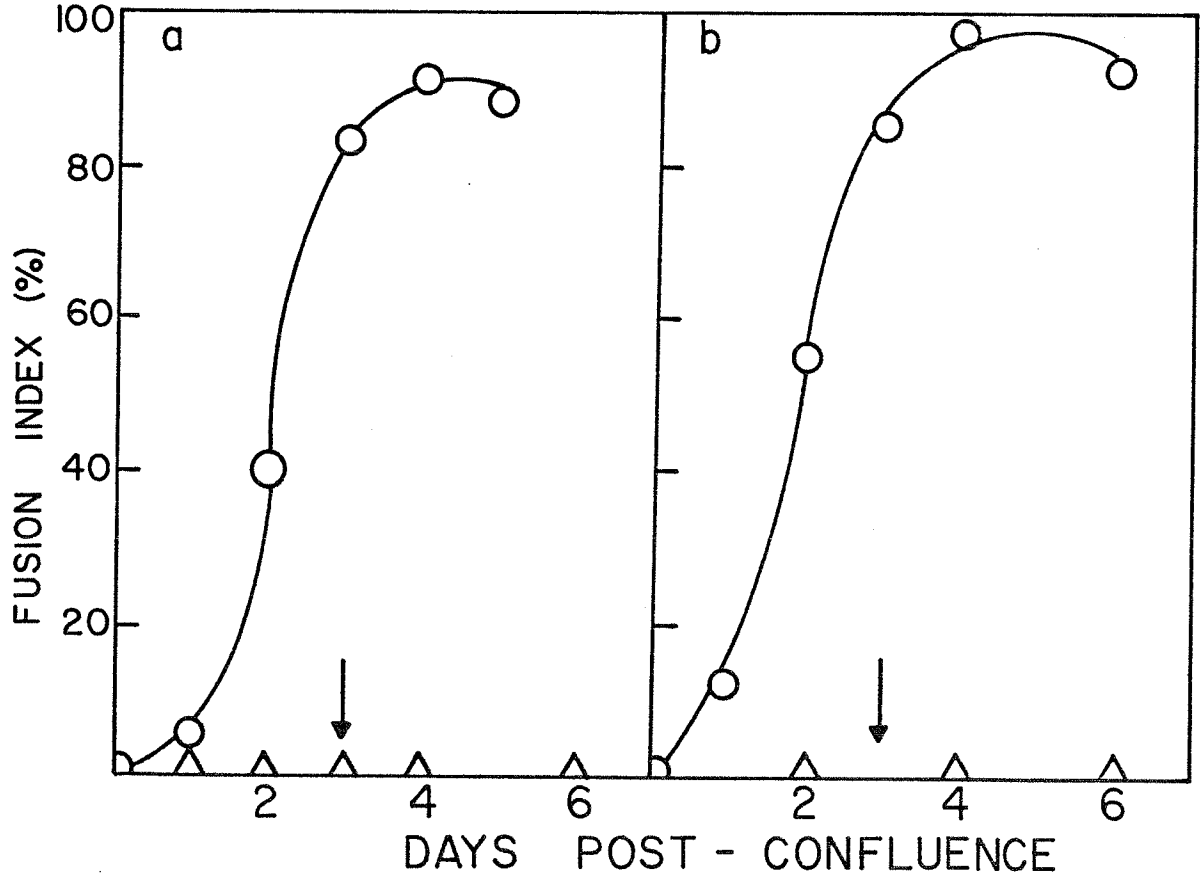
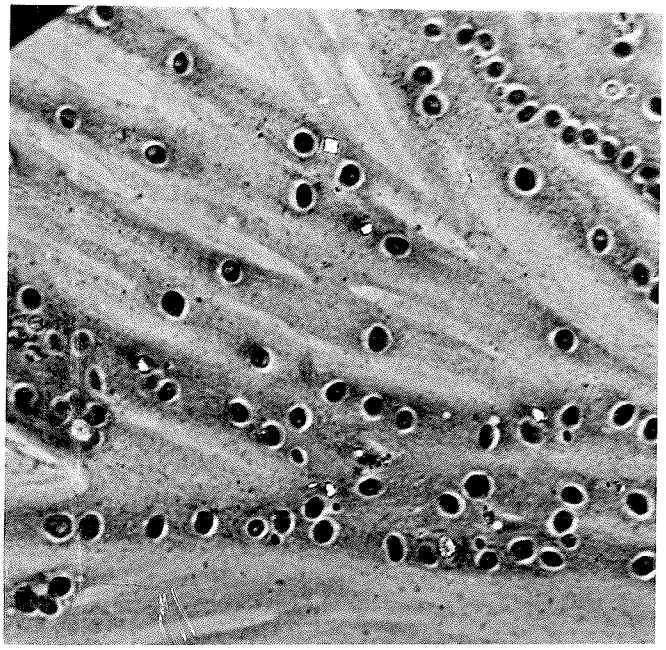
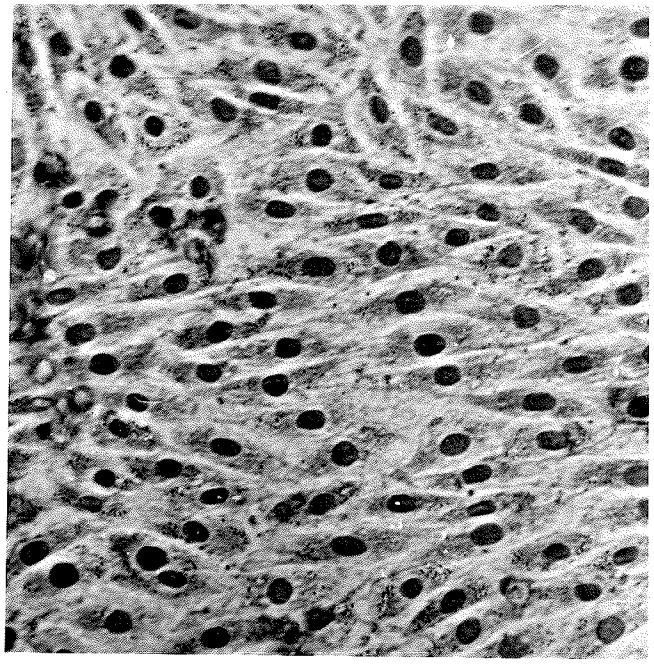

100
80
60
40
20
FUSION INDEX (%)
a
b
2
4
6
2
4
6
DAYS POST - CONFLUENCE

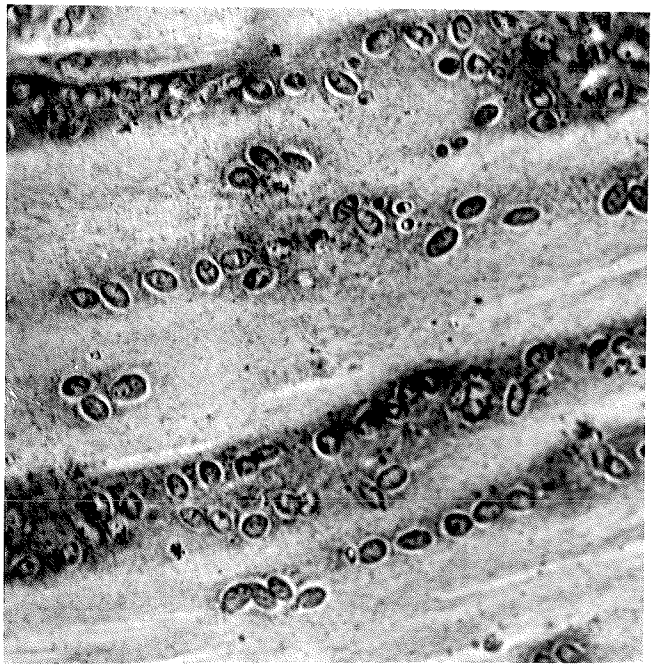
Figure 13: Phase-contrast micrograph of fusing, wild type myoblasts and nonfusing con A-resistant variants. (a) Wild type clone 2; (b) the con A-resistant variant L6CL2V1 selected from it. Phase contrast micrograph of (c) wild type clone 5; and (d) the con A-resistant variant L6CL5V1 selected from it.

a

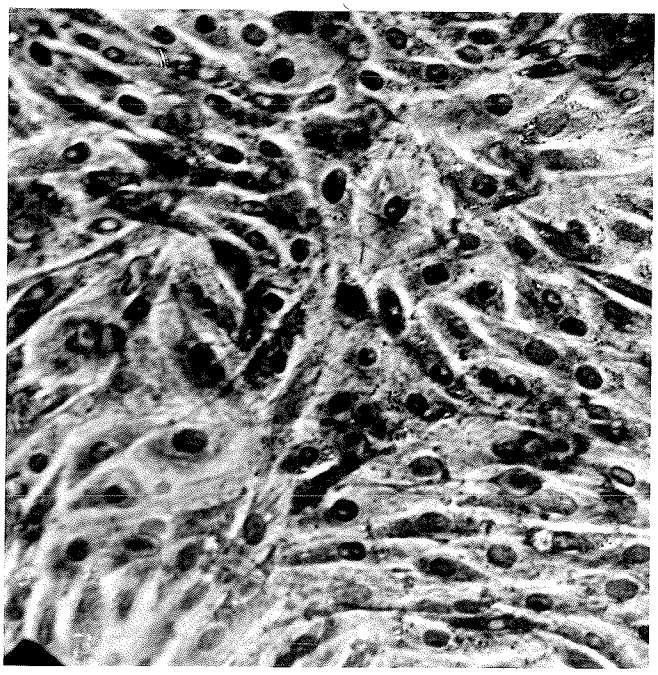


b





c



d

prior to the membrane union event. The result is a confluent layer of apparently contact-inhibited single cells. Above the monolayer, clumps of aggregated cells were often seen floating in the medium, but anchored in some fashion to the monolayer (Figure 14). Similar changes in morphology were observed in con A-resistant Chinese hamster ovary cell lines (Ceri and Wright, 1977). Little overgrowth of the monolayer occurs upon continued feeding; however, the cells are very prone to detachment as a sheet from the substratum upon continued culture past confluence. If these cells are not fed daily with fresh changes of medium, they quickly die. Wild type myoblasts remain firmly attached to the substratum and do not require frequent medium changes to retain their viability. Differentiated cultures have occasionally been maintained for more than seven days beyond the time of maximum fusion.

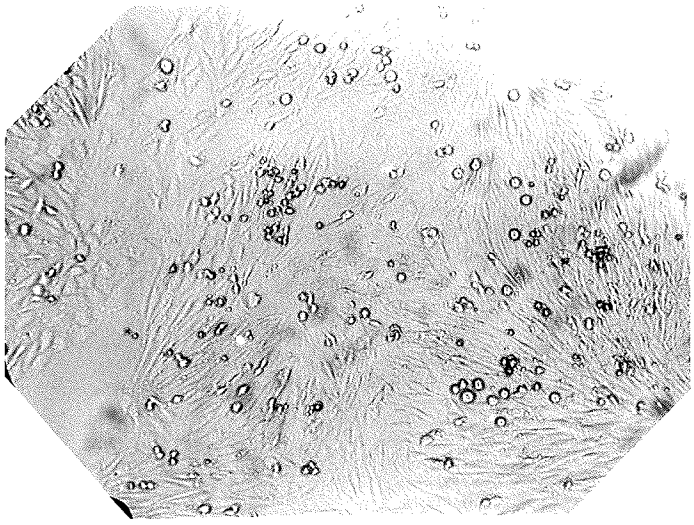
Since myogenesis *in vitro* is often highly dependent on the conditions under which the cells are cultured, several variations on the normal technique of growing the myoblasts were investigated in an attempt to discover if a new set of culture conditions could bring about some morphological differentiation in the con A-resistant myoblasts. These included: growth in αMEM supplemented with foetal bovine, calf or horse serum at high (10%) and low (1%) concentrations; growth at 34°C, 37°C and 39°C; and addition of insulin to the medium, a condition which has been shown to enhance development in L6 myoblasts (Mandel and Pearson, 1974). Under none of these conditions could the beginnings of cell fusion or myotube formation be observed in confluent monolayers of L6CL2V1 cells. A variant L8

Figure 14: Light micrograph of a wild type and a variant myoblast population during logarithmic growth. With daily medium changes, variant myoblasts produce anchored clumps of cells floating above the monolayer. X120.

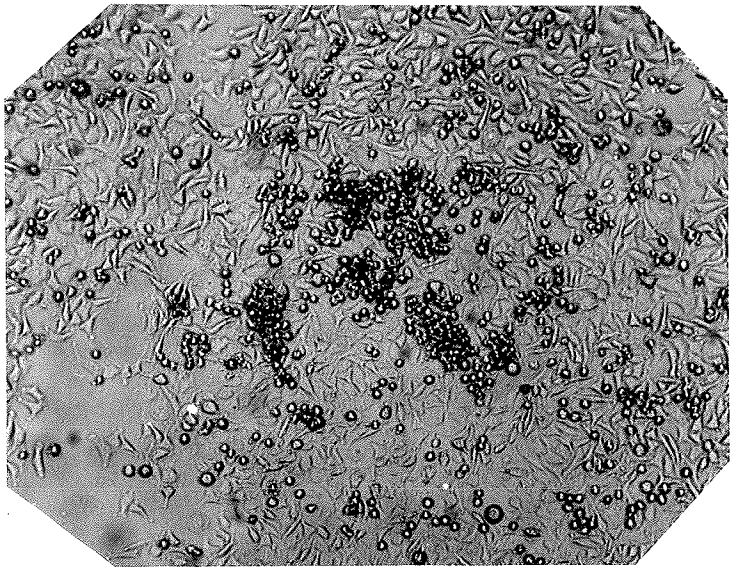
a) Clone 5 WT

b) L6CL5V1

a



b



myoblast line has been described (Yaffe and Saxel, 1977) which has altered differentiation properties in medium containing 10% serum but is able to differentiate normally in medium containing a low serum concentration.

All eight of the con A-resistant lines were examined for the ability to initiate myogenesis under the standard conditions used to induce differentiation. Without exception, con A-resistant myoblasts were unable to form myotubes (Table I).

B Creatine Kinase Activity During Development in Wild Type and Con A-resistant Myoblasts

To determine ifother muscle-specific developmental changes were altered in the con A-resistant myoblasts, the enzyme creatine kinase was assayed throughout the period in which fusion normally takes place (1 to 6 days post confluence, see Figure 12). Figure 15 compares the extent of differentiation attained by wild type clones 2 and 5 myoblast lines and the con A-resistant variants selected from them (L6CL2V1 and L6CL5V1). The variant cells maintain low basal levels of creatine kinase activity (5 to 15 mUnits), but there is a marked increase in enzyme activity accompanying morphological differentiation of the wild type cell lines. Final levels plateau at 125 to 145 mUnits/mg protein.

Table I summarizes the correlation between expression of the con A-resistant phenotype and the loss of the ability to undergo the process of myogenesis. No con A-resistant myoblasts isolated in this study showed a significant increase in creatine kinase levels when compared to their parental wild type cells. The residual expression

Figure 15: Creatine kinase activity after growing myoblasts reach confluence. (a) Specific activity (m Units/mg protein) of wild type, o——o, and L6CL2V1, Δ——Δ, cells. (b) Specific activity of wild type clone 5, o——o, and L6CL5V1, Δ——Δ, cells. Confluence was reached on day one. See Figure 12 for growth conditions.

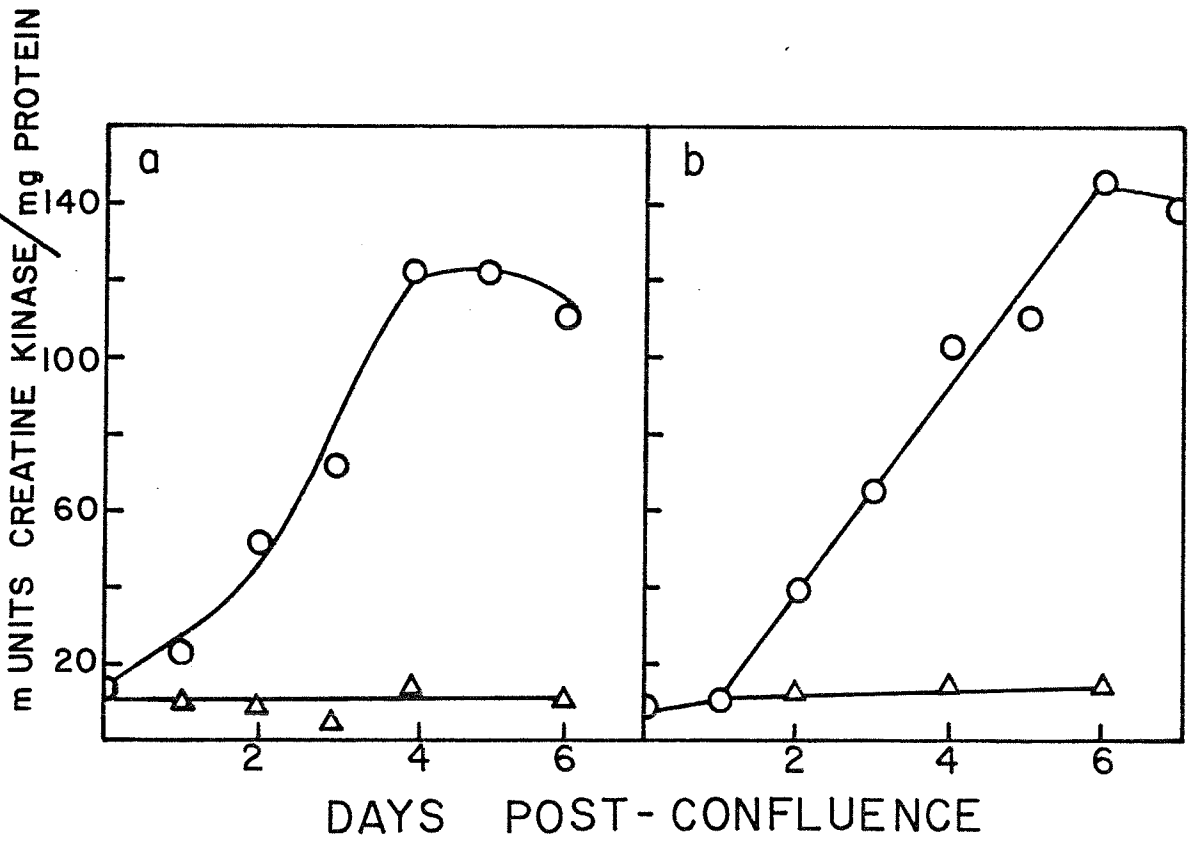

m UNITS CREATINE KINASE/mg PROTEIN
140
100
60
20
a
b
2
4
6
2
4
6
DAYS POST-CONFLUENCE

Table I. A Summary of the Correlation Between Con A-resistance and Fusion Potential.

Myoblast Line	Relative Colony[a] Forming Ability (50 µg/ml con A)	CPK[b] (m Units/mg protein)	Cell[c] Fusion
W.T. clone 1	1.48×10^{-5}	70.0	+
W.T. clone 2	1.5×10^{-5}	109.0	+
W.T. clone 5	9.0×10^{-6}	138.0	+
W.T. clone 6	3.0×10^{-4}	155.0	+
L6CL1V20	1.0	1.3	-
L6CL2V1	4.3×10^{-1}	1.2	-
L6CL5V1	8.0×10^{-1}	6.6	-
L6CL6VI	4.8×10^{-1}	1.6	-
L6CL6V3	1.1×10^{-1}	4.3	-
L6CL6V4	4.1×10^{-1}	0.0	-
L6CL6V5	5.4×10^{-1}	0.0	-
L5CL6V6	4.0×10^{-1}	0.0	-

[a]Colony forming ability in the presence of con A divided by colony forming ability in the absence of con A.

[b]Specific activity was measured 4 to 5 days after confluence on duplicate 60 mm plates. One mUnit of activity = 1 nmole of NADPH formed/minute in the coupled assay system. Preconfluent levels of activity have been subtracted.

[c]Fusion of myoblasts was estimated as + if myotubes containing more than 3 nuclei could be observed in randomly selected fields at 100 x, using phase-contrast optics.

of creatine kinase, where detectable, in the variant lines ranged from 1.0% to 4.0% (L6CL6V1 and L6CL5V1 respectively) of wild type levels of the enzyme activity.

C Clonal Variation of Myogenic Potential in Wild Type Myoblasts

The frequency at which non-fusing variants spontaneously arise from a freshly cloned wild type L6 myoblast population was investigated by randomly isolating subclones of the population, growing each subclone to a monolayer and observing their ability to initiate membrane fusion and to produce the muscle-specific CPK activity. The clones were isolated from a wild type population in the same way that the con A-resistant variant selection experiments were performed. Nineteen subclones were tested in this manner and Table 2 shows that all of them exhibited a fusion-competent phenotype and significant increases in CPK specific activity occurred during differentiation. Furthermore, an additional 57 subclones were isolated and tested only for the ability to initiate membrane fusion and were found to be fusion-competent. These studies show that the occurrence of non-fusing cells in a freshly cloned population, like those used to select con A-resistant myoblasts, is less than 1.3%. Therefore, the possibility of consecutively recovering four non-fusing variants by independent selections is less than 2.8×10^{-8}. If we assume that the five variants isolated from wild type clone 6 arose from independent genetic events, then the possibility of selecting a total of eight independent non-fusing cell lines in a row would be less than approximately 8 in 10^{16} attempts.

Table II. Myogenesis with Clones of L6 Myoblasts.

Clone No.	CPK[a]	Fusion[b]	Clone No.	CPK	Fusion
1	79.5	+	11	33.0	+
2	105.0	+	12	33.0	+
3	61.0	+	13	39.5	+
4	78.5	+	14	67.0	+
5	82.0	+	15	49.5	+
6	110.0	+	16	37.0	+
7	69.0	+	17	50.0	+
8	62.0	+	18	77.0	+
9	76.0	+	19	32.0	+
10	69.0	+			

[a]Creatine phosphokinase activity was expressed as mUnits/mg protein. Preconfluent myoblasts expressed 5 to 10 mUnits of CPK/mg protein.

[b]Fusion of myoblasts was considered + if myotubes containing more than 3 nuclei could be observed in randomly selected fields at 100 x, using phase-contrast optics. The CPK and fusion estimates were obtained 4 to 5 days post confluence and represent plateau levels (e.g., see Figure 4).

The observation that all the con A-resistant myoblast lines are fusion-negative, strongly supports the view that the con A-resistant phenotype and the loss of myogenic potential are not independent.

In addition, several spontaneous nonfusing cell lines, unselected for drug or lectin resistance, were isolated and tested for their sensitivity to con A. Three lines were derived by cloning single cells which survive several serial passages in which the monolayer is allowed to differentiate. Cells remaining undifferentiated were used as the inoculum for the next passage in the series. Two other cell lines were obtained by selecting cells resistant to the anti-tumor drug hydroxyurea which were shown to be nonfusers (Creasey *et al.*, 1982; Wright *et al.*, 1982). Examination of confluent monolayers for any myotube formation revealed that none of these cell lines could initiate differentiation. The relative plating efficiency of these cell lines was then compared to L6CL2V1 cells at a single concentration (60 µg/ml) of con A (Table III). The myogenesis-defective cells were not resistant to the cytotoxic effect of the lectin, suggesting that a loss of myogenic potential in the L6 wild type lines does not always result in an altered sensitivity to con A. Rather, it appears that the biochemical lesion responsible for con A-resistance is one of fundamental importance to the myogenic process, and that this lesion is only one of many unknown defects which also preclude the differentiation program.

Table III. Relative Plating Efficiency of Nonfusing Myoblasts in Con A Supplemented Medium.

Cell line	R.P.E.[a]
$L_6CL2\ V_1$	9.8×10^{-1}
HU^R11c[b]	4.5×10^{-5}
HU^R4c	5.2×10^{-4}
non fuser 1	$<10^{-5}$
non fuser 2	$<10^{-5}$
non fuser 3	$<10^{-5}$

[a]The relative plating efficiency was determined at a con A concentration of 60 µg/ml in αMEM supplemented with 10% foetal bovine serum. Five plates containing 10^5 cells of each line were scored.

[b]Cell lines resistant to hydroxyurea were isolated, after a two step selection in 2 mM drug, by their ability to form colonies in 0.5 mM hydroxyurea. Wild type D_{10} = 0.35 mM; HU^R11c D_{10} = 1.56 mM; HU^R4c D_{10} = 2 mM

[c]Nonfusing myoblasts were selected at random (i.e., *not* by con A) after 3 serial passages in which the confluent monolayer was allowed to fully differentiate.

Section III Further Characterization of the Cellular Phenotype in Wild Type and Con A-resistant Myoblasts

The experiments described in the following pages were initiated with two purposes in mind. The first was to compare the phenotype of the con A-resistant myoblast cells with the phenotype of the con A-resistant Chinese hamster ovary cells previously isolated and described in this laboratory (Wright, 1979; Wright *et al.*, 1980). It was suspected that if significant alterations in membrane-associated interactions had occurred, then a useful selection scheme might be designed to recover a revertant phenotype, as had been done for the hamster cell lines. Secondly, the expression of the transformed phenotype, in which cells become aberrant in basic mechanisms of growth and differentiation is well known to cause extinction of differentiated functions (Braun, 1975). Since the con A-resistant myoblasts were shown to be defective in their ability to undergo myogenesis, it seemed possible that cellular transformation might be an underlying cause. With this in mind, several *in vitro* correlates of the transformed phenotype were examined for their expression in con A-resistant myoblasts.

A Karyology

Characterization of the model chromosome number is an important aid in establishing the identity of a cell line in culture (Worton

and Duff, 1979). The importance of such identification has been realized in cases where cross-contamination of cell lines has occurred, resulting in incorrect interpretations of experimental results (Nelson-Rees and Flandermeyer, 1976). Chromosomal changes are often concomitant of neoplasia although their relationship to the initiation of transformation is unknown (DiPaolo and Popescu, 1976).

Metaphase chromosome spreads were made from exponentially growing myoblasts after arresting mitosis for a period of 2 to 3 hours using colchicine. An average of 60 to 70 spreads were examined and individual chromosomes were enumerated. Typical chromosomal appearance in wild type clones 2 and 5, and the con A-resistant lines selected from them are shown in Figures 16 and 17. The chromosome numbers of the wild type lines were found to be widely distributed, from 60 to 74 with the mode at 71. Normal rat somatic cells have a diploid number of 42 chromosomes, thus the L6 lines appear to be hypotetraploid (Figures 18, 19). L6CL2V1 and L6CL5V1 lines are also hypotetraploid with model numbers of 70 and 66 respectively. No significant changes in chromosome number could be detected between the wild type and con A-resistant cell lines. Therefore, gross changes in chromosome numbers between wild type and con A-resistant myoblasts are not likely to play a role in the acquisition of the lectin resistant phenotype.

Figure 16: Frequency histograms of metaphase chromosome counts for wild type clone 2 and L6CL2V1 cells. (a) Wild type; (b) variant.

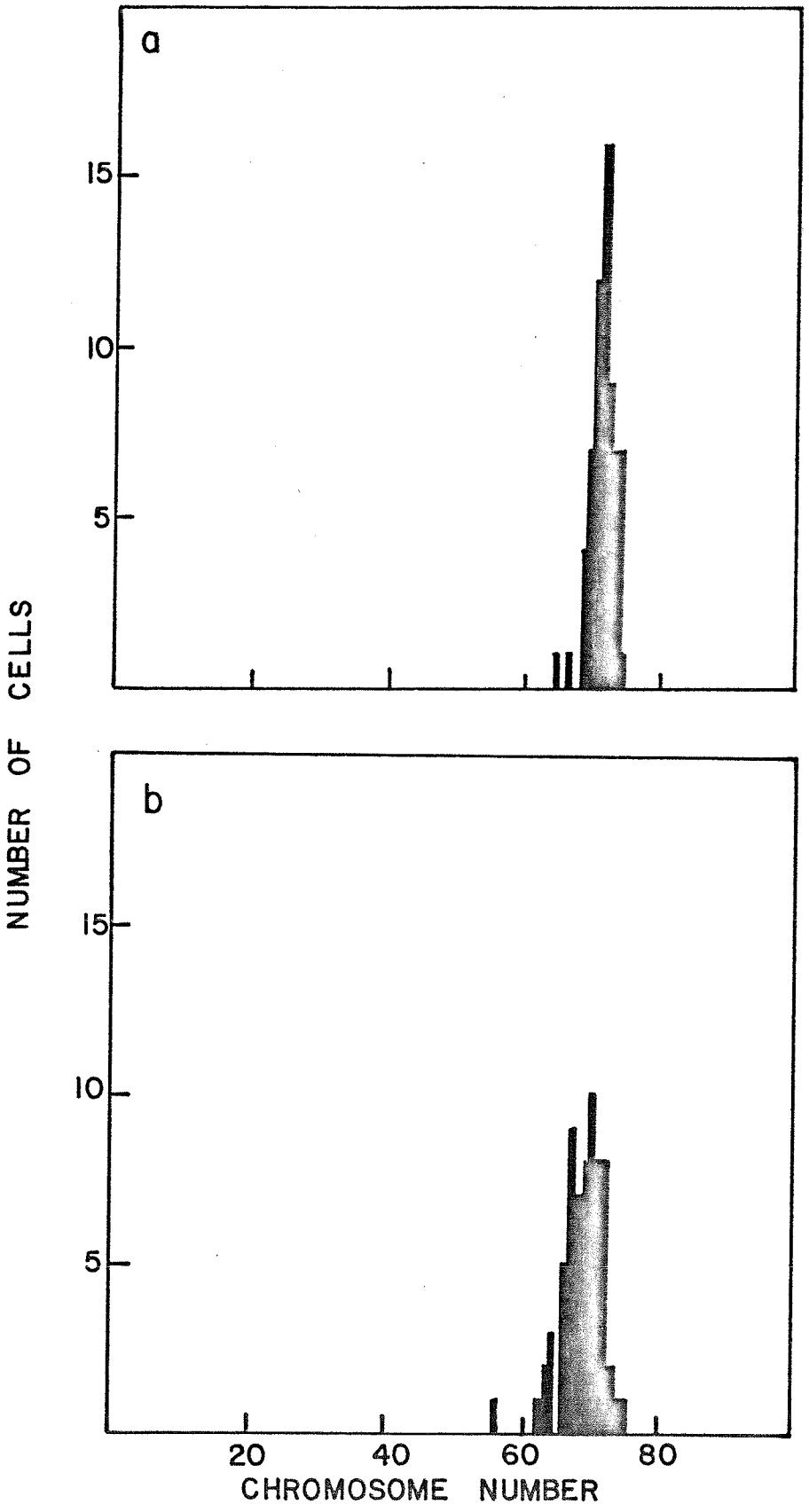

a
b
15
10
5
15
10
5
NUMBER OF CELLS
20
40
60
80
CHROMOSOME NUMBER

Figure 17: Frequency histograms of metaphase chromosome counts for wild type clone 5 and L6CL5V1 cells. (a) Wild type; (b) variant.

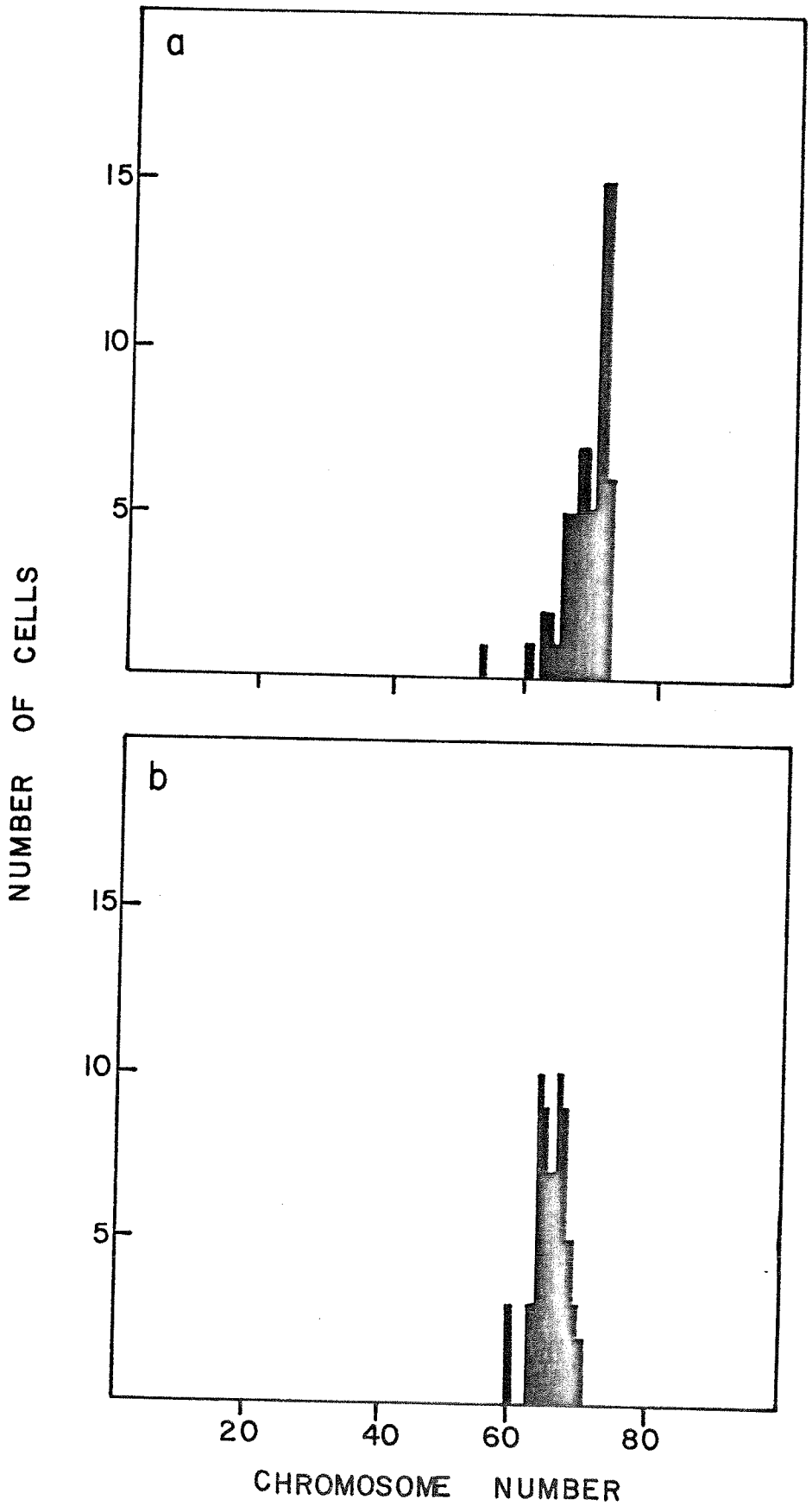

a
b
15
10
5
15
10
5
NUMBER OF CELLS
20
40
60
80
CHROMOSOME NUMBER

Figure 18: Phase-contrast micrograph of metaphase chromosome spreads from wild type clone 2 and L6CL2V1 cells. (a) Wild type; (b) variant. X 1880.

a

b

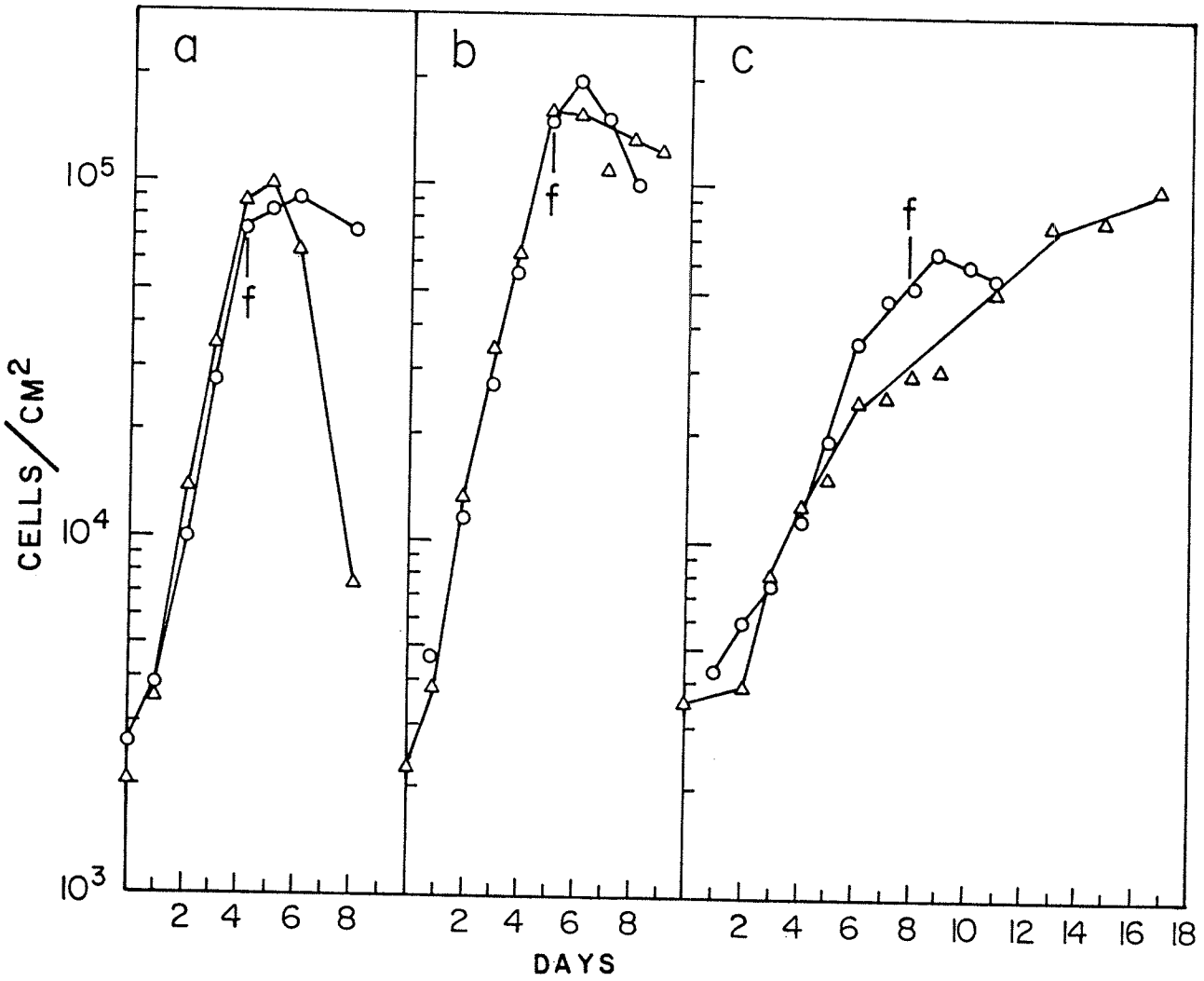
Figure 19: Phase-contrast micrograph of metaphase chromosome spreads from wild type clone 5 and L6CL5V1 cells. (a) Wild type; (b) variant. X 1880.

B Saturation Density

It was noticed that during continued incubation of the con A-resistant myoblasts without adding fresh growth medium leads to cell death and eventually detachment, whereas the wild type myoblasts continue to remain attached to the culture plate as myotubes (Figure 20a). However, Figure 20b shows that daily medium changes eliminated the tendency of the variant cells to die upon reaching confluence, and under these conditions the final saturation density of both wild type and variant cells reached approximately 2×10^6 cells/cm^2. As transformed cells often have reduced requirements for serum, the growth of con A-resistant myoblasts was examined in medium supplemented with 1% calf serum. Growth with daily changes of low serum-medium resulted in a three-fold reduction in the saturation density of wild type cells while the variant myoblasts continued to grow slowly past confluence with a 1.6-fold reduction in saturation density (Figure 20c). Furthermore, even after 17 days of growth in 1% calf serum, the variant myoblasts were not observed to initiate membrane fusion, whereas the wild type cells began to differentiate at cell saturation density, preventing further increases in cell number. Therefore, at low serum concentrations, the variant cells are capable of slow but extended proliferation past the cell density at which the wild type begins to differentiate, but if fresh medium is not continuously supplied, non-viable variant cells detach from the culture plate.

Growth rates during the exponential phase in 10% serum were identical for wild type clone 2 and L6CL2V1 cells. The doubling

Figure 20: Growth curves at 37°C in medium containing calf serum. After each day of incubation, cells were removed from the surface of duplicate plastic plates with 0.2% buffered trypsin solution and an aliquot was counted with an electronic particle counter (Coulter Electronics Ltd.). Cell growth in (a) 10% serum on 100 mm culture dishes without additions of fresh medium during the course of the experiment; (b) 10% serum with daily changes of medium; (c) 1% serum on 60 mm plates with daily changes of medium. o——o, wild type clone 2; Δ——Δ, L6CL2V1. → , time of first appearance of progressively growing multinucleated myotubes in the wild type clone 2 cultures.


a
b
c
f
f
f
10^5
10^4
10^3
CELLS/CM^2
2 4 6 8
2 4 6 8
2 4 6 8 10 12 14 16 18
DAYS

time was estimated to be 16 hours in each case. However, exponential growth in 1% serum was much slower, with a doubling time of 28 to 30 hours for these cell lines. In addition, a period of very slow growth was observed just before saturation was attained in 1% serum. In this portion of the growth curve, doubling times were estimated to be 80 to 100 hours.

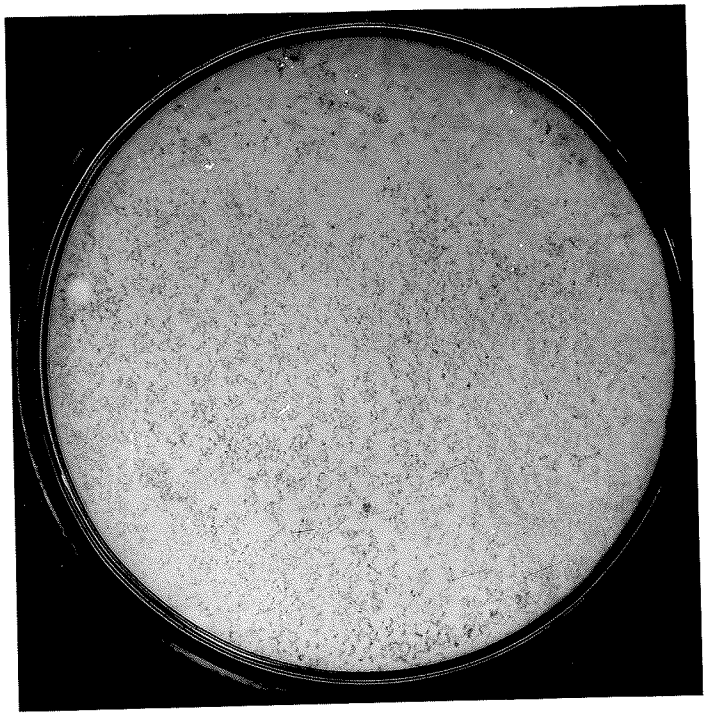
C Plasminogen Activator Synthesis

Transformed cells are often distinguished by their ability to hydrolyse fibrin, although exceptions are known (e.g. Barrett *et al.*, 1980). Two components have been recognized as playing a role in this activity: one being present in the serum supplement of culture media and the other released by cells following the transformation event (Unkeless *et al.*, 1973a; Ossowski, 1973). The serum factor has been identified as the zymogen, plasminogen (Quigley *et al.*, 1973) while the cell-released factor is an arginine specific protease of molecular weight 38,000d (Unkeless *et al.*, 1973b).

Wild type clones 2 and 5 were examined for their ability to produce this protease by visualizing hydrolysis of casein precipitate in agar overlayers, after activation of plasminogen (Figure 21). The cleared areas within the precipitate began to appear over the colonies after about 24 hours of incubation. Removal of serum from the agar overlayer totally prevented casein hydrolysis confirming that the assay was specific for plasminogen and not a cell-derived proteolytic activity. Table IV compares the extent of plasminogen activator synthesis, expressed as the percentage of colonies positive for the

Figure 21: Plasminogen activator synthesis by wild type and con A-resistant myoblast colonies. (a) Wild type clone 2; (b) L6CL2V1. Each 100 mm plate contains approximately 100 colonies of about 200 cells each overlayered with agarose containing casein and 10% human serum. The agar overlayer was photographed after 36 hours of incubation at $37^{o}C$.

a



b

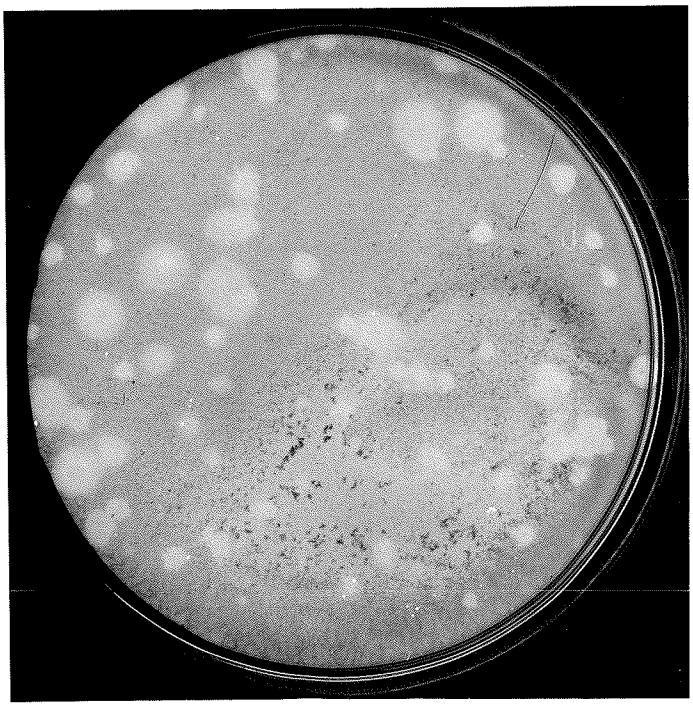


Table IV. Plasminogen Activator Synthesis by Wild Type and Con A-resistant L6 Myoblasts.

	Wild type	Variant
Clone 2	10.1%[a]	58.0%
Clone 5	28.1%	9.9%

[a]The amount of plasminogen activator activity is expressed as the percentage of colonies hydrolysing casein. The average value of five plates, each containing 100-150 colonies has been calculated for each cell line.

activity. Clearly, both wild type and variant L6 myoblasts produce plasminogen activator, but there is no correlation between that production and the con A sensitivity of the cell which is producing it. Both increased (L6CL2V1) and decreased (L6CL5V1) activity were seen when compared to the wild type clone from which it was selected. Since all four lines tested were different in plasminogen activator expression, clonal variation, unrelated to any effects of the lesion which results in con A-resistance, may be the cause of the different levels observed.

D Growth in Sloppy Agar

As transformed cells often have reduced requirements for substrate attachment during culture *in vitro* (Goldberg, 1974), the growth of wild type and variant myoblasts in soft agar was examined. Colonies of more than 20 cells were scored after 14 days of growth and the results are presented in Table V. Wild type clone 2 myoblasts form few colonies under these conditions, in keeping with their normal phenotype (average plating efficiency 0.014%). L6CL2V1 cells however show a slightly higher capacity to grow in soft agar since small colonies were observed on all plates incubated with these cells (average plating efficiency 0.58%). Both of these plating efficiencies are low however when compared with wild type Chinese hamster ovary cells which form colonies under the conditions used in this study at a plating efficiency of 24%.

Table V. Growth of Wild Type and Con A-resistant Myoblasts in Soft Agar.

Cell line	Cells plated	Colonies Counted*	Plating Efficiency
wild type clone 2	1×10^3	0	0.0
	5×10^3	1	2×10^{-4}
	1×10^4	3	3×10^{-4}
	5×10^4	2	4×10^{-5}
	1×10^5	1	1×10^{-5}
			Average = 1.4×10^{-4}
L6CL2V1	1×10^3	10	1×10^{-2}
	5×10^3	30	6×10^{-3}
	1×10^4	39	4×10^{-3}
	5×10^4	157	3×10^{-3}
			Average = 6×10^{-3}
wild type Chinese hamster ovary cells	1×10^3	240	2.4×10^{-1}

*Only colonies of greater than 20 cells were counted after 2 weeks of growth at 37°C. Chinese hamster ovary cell colonies grew quickly and were counted at 7 days.

E Cytotoxicity of Cell-Surface Active Compounds

If significant changes have occurred in the structure of glycoproteins it is likely that membrane functions such as permeability, transport of metabolites and substrate attachment would be altered. These effects can be measured by determining the sensitivities of myoblasts to cytotoxic levels of agents having major effects at the cell surface. Wright (1973) and Ceri and Wright (1977), have shown that Chinese hamster ovary cells selected for con A-resistance show increased sensitivities to such agents. Many of the same compounds were examined for their D_{50} concentrations, as well as other agents such as lectins or glycoprotein biosynthesis inhibitors (Figures 22-24), including: the cytotoxic lectins ricin, WGA and PHA; the glycoprotein biosynthesis inhibitors 25-hydroxycholesterol, compactin, 2-deoxyglucose, bacitracin (Schwarz and Datema, 1980), oubain, an inhibitor of Na^+/K^+ ATPase activity, and two denaturants, phenol and phenethyl alcohol. Cytotoxicity of hydroxyurea, an antitumor drug specific for the intracellular enzyme ribonucleotide reductase (Wright *et al.*, 1981) was examined as a test of general membrane permeability. Calcium chloride was used to increase the ionic strength of the medium.

L6CL2V1 cells were more sensitive to several agents such as the lectins Ricin, and WGA. Slightly increased sensitivity was also seen for WGA, phenol and oubain when compared to wild type clone 2 myoblasts. These differences were not however, large enough to exploit as an efficient revertant selection scheme. For example,

Figure 22: Relative colony forming ability of wild type and con A-resistant myoblasts in the presence of various concentrations of oubain, hydroxyurea, Ca^{++} and 25-hydroxy-cholesterol. These chemical agents were added to αMEM supplemented with 5% foetal bovine serum in 100 mm culture dishes. Each dish was seeded with 1000 cells and incubated at 37°C for 7 to 10 days. o——o, wild type clone 2; Δ——Δ, L6CL2V1.

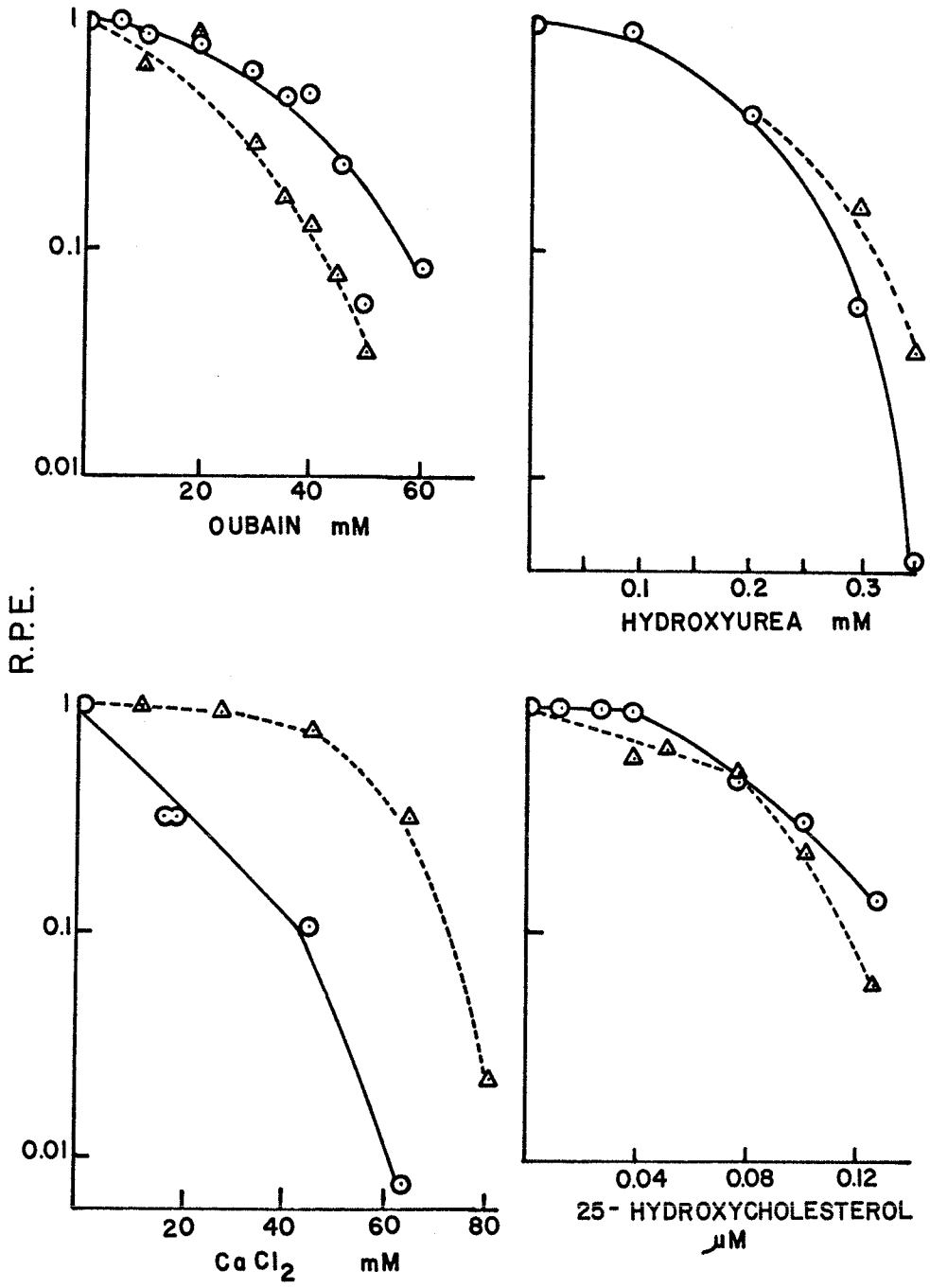

R.P.E.
1
0.1
0.01
20
40
60
OUBAIN mM
0.1
0.2
0.3
HYDROXYUREA mM
1
0.1
0.01
20
40
60
80
Ca Cl2 mM
0.04
0.08
0.12
25- HYDROXYCHOLESTEROL
μM

Figure 23: Relative colony forming ability of wild type and con A-resistant myoblasts in the presence of various concentrations of 2-deoxyglucose, bacitracin, compactin and ricin. See Figure 22 for growth conditions. o——o, wild type clone 2; Δ——Δ, L6CL2V1.

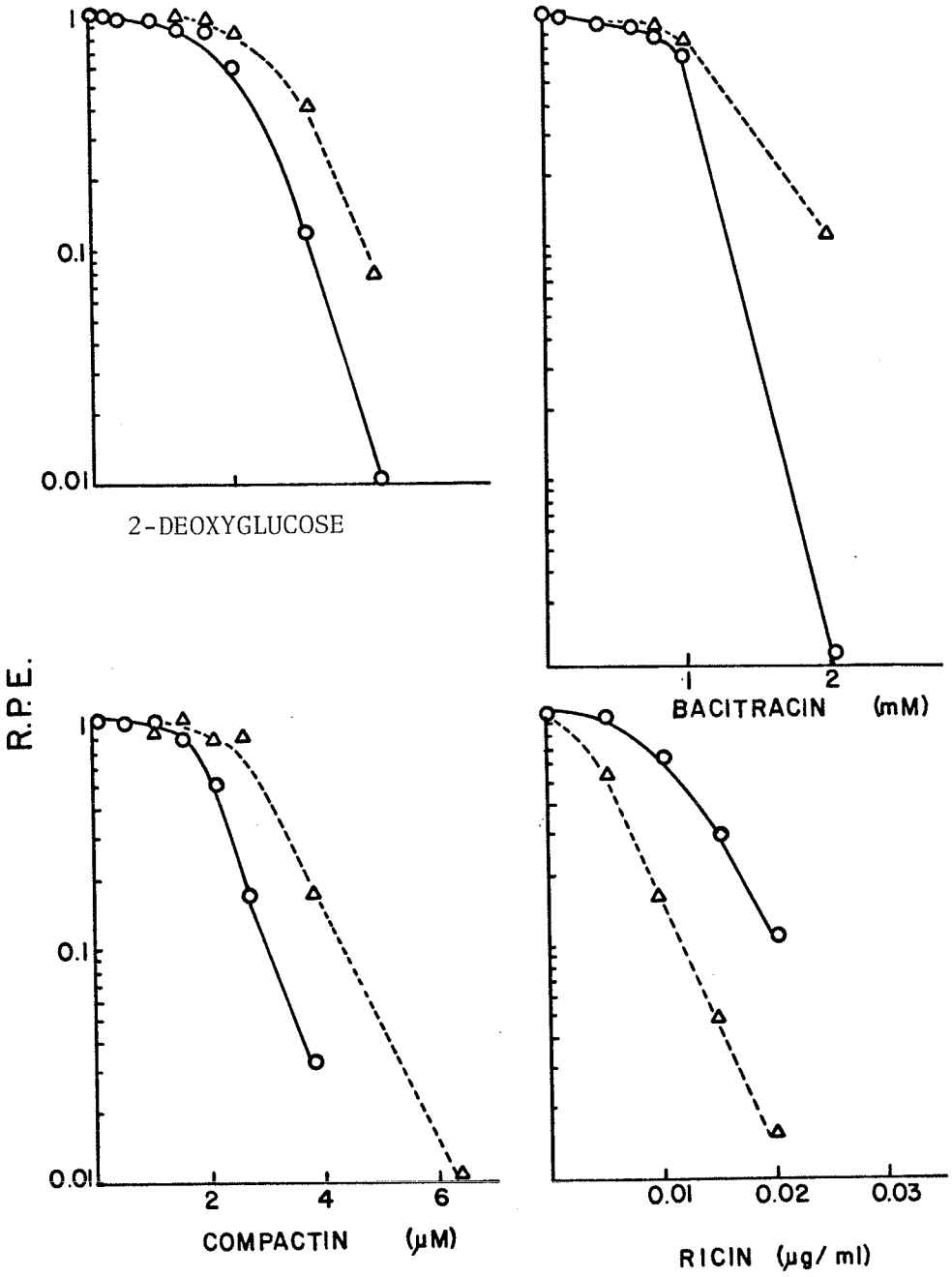
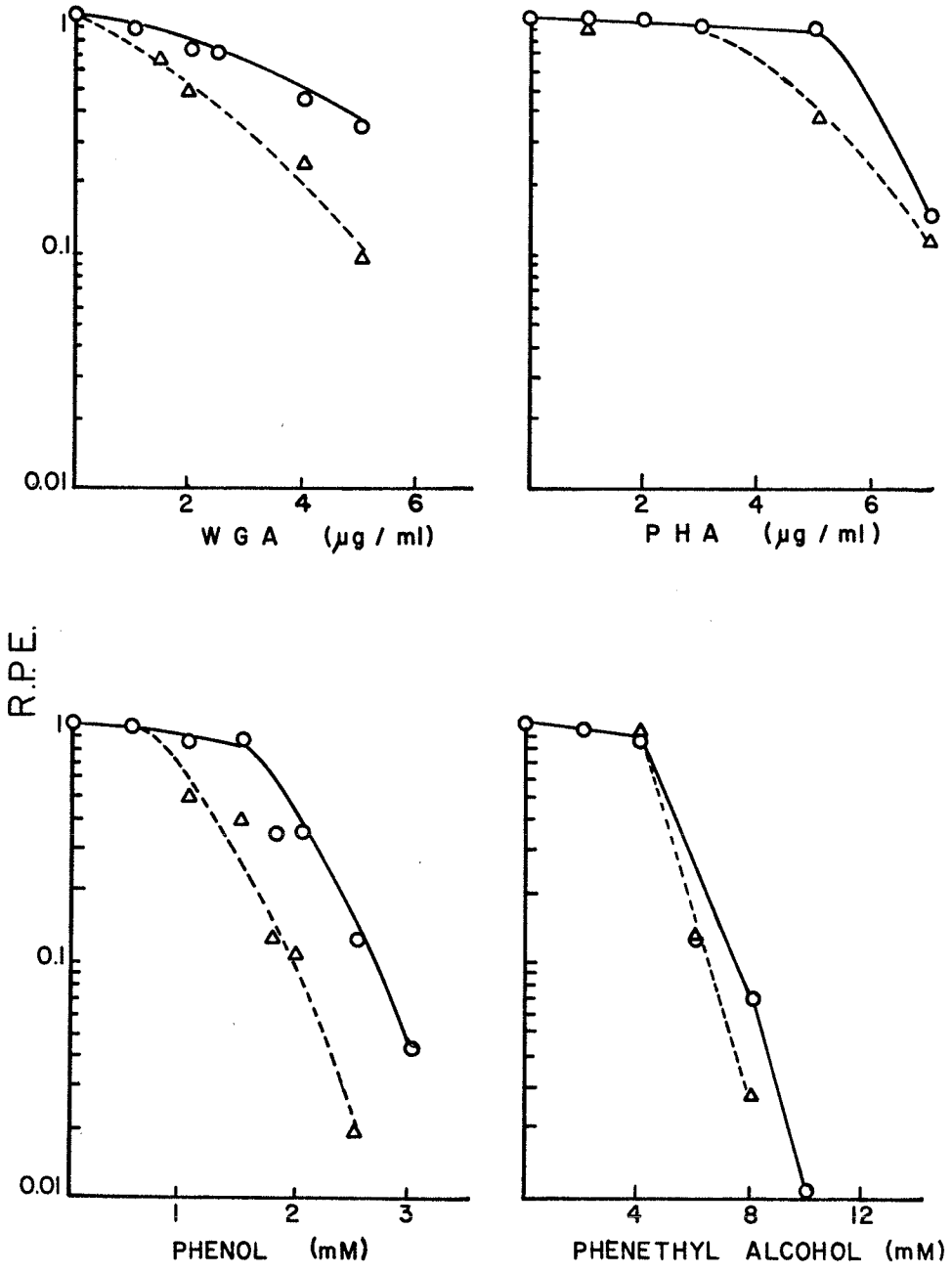

R.P.E.
1
0.1
0.01
2-DEOXYGLUCOSE
1
2
BACITRACIN (mM)
1
0.1
0.01
2
4
6
COMPACTIN (μM)
0.01
0.02
0.03
RICIN (μg/ml)

Figure 24: Relative colony forming ability of wild type and con A-resistant myoblasts in the presence of various concentrations of WGA, PHA, phenol and phenethylalcohol. See Figure 22 for growth conditions. o——o, wild type clone 2; Δ——Δ, L6CL2V1.


R.P.E.
1
0.1
0.01
2
4
6
W G A (µg / ml)
P H A (µg / ml)
1
2
3
PHENOL (mM)
4
8
12
PHENETHYL ALCOHOL (mM)

ricin was the most cytotoxic agent tested for variant cells, when compared to the parental line, but at a concentration of lectin in which over 50% of the wild type cells may form colonies, about 17% of the variant cells also form colonies.

Sensitivity of variant myoblasts to the remaining agents was either unaltered or it was slightly reduced when compared to wild type myoblasts. D_{50} values are summarized in Table VI.

F Temperature Sensitivity

Chinese hamster ovary cell lines selected for con A-resistance also display a sensitivity to growth at elevated temperature (Wright, 1973, 1975; Ceri and Wright, 1977; Cifone *et al.*, 1979). This property has been an advantage for the selection of revertant lines expressing a con A-sensitive phenotype. Consequently, the effect of temperature on colony formation by wild type myoblast clones 2 and 5 and the con A-resistant lines selected from them was examined. Colony formation was reduced to less than 0.1% at 40.5°C in clone 5 wild type and L6CL5V1 while both clone 2 wild type and L6CL2V1 reached this level at 41.5°C (Table VII). It appears that the lesion responsible for producing a relative resistance to con A in the myoblasts has no significant effect on their growth at higher temperature.

Table VI. Sensitivity of Wild Type and Con A-resistant Myoblasts to Various Cytotoxic, Membrane-active Agents.

Agent	D_{50} Wild type clone 2	D_{50} L6CL2VI
oubain	32 mM	22 mM
hydroxyurea	0,17 mM	0.17 mM
$CaCl_2$	11 mM	53 mM
25-hydroxycholesterol	0.075 µM	0.075 µM
2-deoxyglucose	1.0 mM	1.4 mM
bacitracin	1.0 mM	1.2 mM
compactin	2.0 µM	2.7 µM
ricin	0.012 µg/ml	0.005 µg/ml
WGA	3.6 µg/ml	2.0 µg/ml
PHA	5.6 µg/ml	4.5 µg/ml
phenol	1.75 mM	1.0 mM
phenethylalcohol	4.6 mM	4.6 mM

Table VII. Growth of Wild Type and Con A-resistant Myoblasts at Elevated Temperature.

Temperature	Wild type clone 2	L6CL2V1	Wild type clone 5	L6CL5V1
37°C	100	100	100	100
39°C	-	-	62	80
39.5°C	100	62	-	-
40.5°C	77	26	0	0
41.5°C	0	0	-	-

Growth at each temperature is expressed as the percentage of cells that form colonies. 200 cells were plated on each 100 mm plastic culture dish, and the average of three dishes was calculated.

Section IV Alterations of Cell Surface Glycoproteins on Surface Membranes of Con A-resistant Myoblasts

A Con A Binding to Myoblast Surfaces

The resistance of the variant myoblast lines to the cytotoxic effects of con A and possibly their inability to initiate myogenesis may result from surface glycoproteins of altered structure, which reduces their ability to bind the cytotoxic lectin to the same extent as the surface glycoproteins of wild type myoblasts. Wright and Ceri (1977a,b) have previously shown that con A-resistant hamster lines bind significantly less con A if measured at $4^{o}C$. This method prevents direct endocytosis of the surface bound lectin, which can mask subtle differences in that binding. Preliminary studies were carried out with wild type clone 2 and L6CL2V1 cells to determine if binding saturation could be achieved under conditions in which endocytosis was likely to be inhibited. A time course binding study was carried out at $4^{o}C$ in the presence of 10 mM of the metabolic inhibitor sodium azide (Figure 25). It was observed that the amount of lectin bound approaches saturation at 20 minutes. This time period was routinely used in all subsequent binding studies.

Several buffer solutions were examined for their effect on binding. Although binding of con A occurred in 0.85% saline, PBS and 0.01 M Tris buffered saline, pH 7.0, supplemented with $10^{-3}M$ Ca^{++}, Mn^{++}, Mg, the greatest binding was obtained in 0.85% saline without buffer, pH 7.0 (Table VIII). All binding studies were subsequently performed

Figure 25: [^{3}H] con A binding to wild type and con A-resistant myoblasts increases with time. o——o, wild type clone 2; Δ——Δ, L6CL2V1. Binding (100 μg/ml con A) was assayed in PBS containing 10 mM NaN_3 at 4°C after the myoblasts had been grown to confluence in medium supplemented with 10% foetal bovine serum.

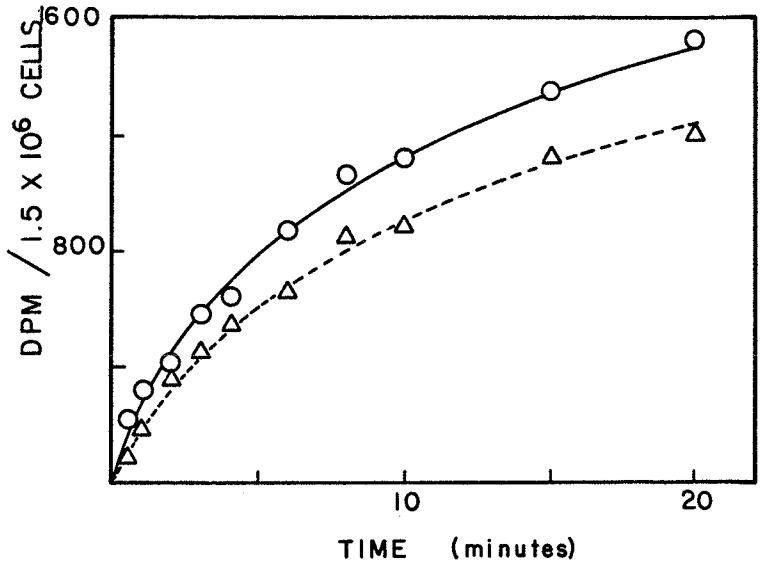

DPM / 1.5 X 10^6 CELLS
1600
800
10
20
TIME (minutes)

Table VIII. Con A Binding to Myoblast Cell Surfaces - A Comparison of Various Buffers.

(1) Growth in 10% calf serum	L6CL2V1	Clone 2 wild type
a) saline, 0.85%	3802 dpm/ 1.5×10^6 cells	4775
b) PBS	3161	4168
c) 0.01 M TBS, pH 7.0 0.001 M Ca^{++}, Mg^{++}, Mn^{++}	2558	3106
(2) 16 hours in medium with no serum supplement	L6CL2V1	Clone 2 wild type
a) saline, 0.85%	1395 dpm/ 1.5×10^6 cells	2887
b) PBS	1416	2592
c) 0.01 M TBS, pH 7.0 0.001 M Ca^{++}, Mg^{++}, Mn^{++}	1393	2343

All solutions contain 0.85 M NaCl and 0.01 M NaN_3.

using this solution. Under these conditions, nonspecific binding in the presence of 0.2 M α methyl-D-mannoside was measured to be 3 to 5% of total cell surface binding.

The results of these initial experiments indicated that the con A-resistant mutant cell line bound significantly less of the lectin than did the wild type clone 2 cells. Many cell lines selected for lectin resistance exhibit decreased lectin binding characteristics (Wright *et al.*, 1980; Wright, 1979). Therefore, to determine if this was true of the con A-resistant myoblasts selected in this study, all were compared with their parental wild type lines.

When logarithmically growing cells were incubated with [^{3}H] con A at all concentrations of from 0.25 to 10 μg/ml, a reproducible fraction of the soluble lectin became associated with the cells upon the culture dish surface (Figure 26). The relative binding to the variant lines averages 50% of that bound to the normal myoblasts at these concentrations, and the relationship of bound to free lectin appears to be a straight line in this region.

In order to investigate the kinetics of con A binding in more detail, one of the resistant myoblast lines (L6CL2V1) and its parental wild type population was examined for lectin binding over a 4000-fold range of concentrations (0.25 to 1000 μg/ml) in several different experiments. As shown in Figure 27, saturation binding was observed and the binding curves appeared to be hyperbolic. When the data obtained with the variant cell line was analyzed by the Scatchard

Figure 26: [^{3}H] con A binding to intact wild type and con A-resistant myoblasts at low concentrations of the lectin. ●——●, wild type clone 2 and, o——o, L6CL2V1 myoblasts. (a) Wild type clone 2 and L6CL2V1; (b) wild type clone 5 and L6CL5V1; (c) wild type clone 1 and L6CL1V20; (d) wild type clone 6 and L6CL6V6. Binding was measured at 4^{o}C in saline containing 10 mM NaN_3 after cells were grown to confluence in medium supplemented with 10% foetal bovine serum.

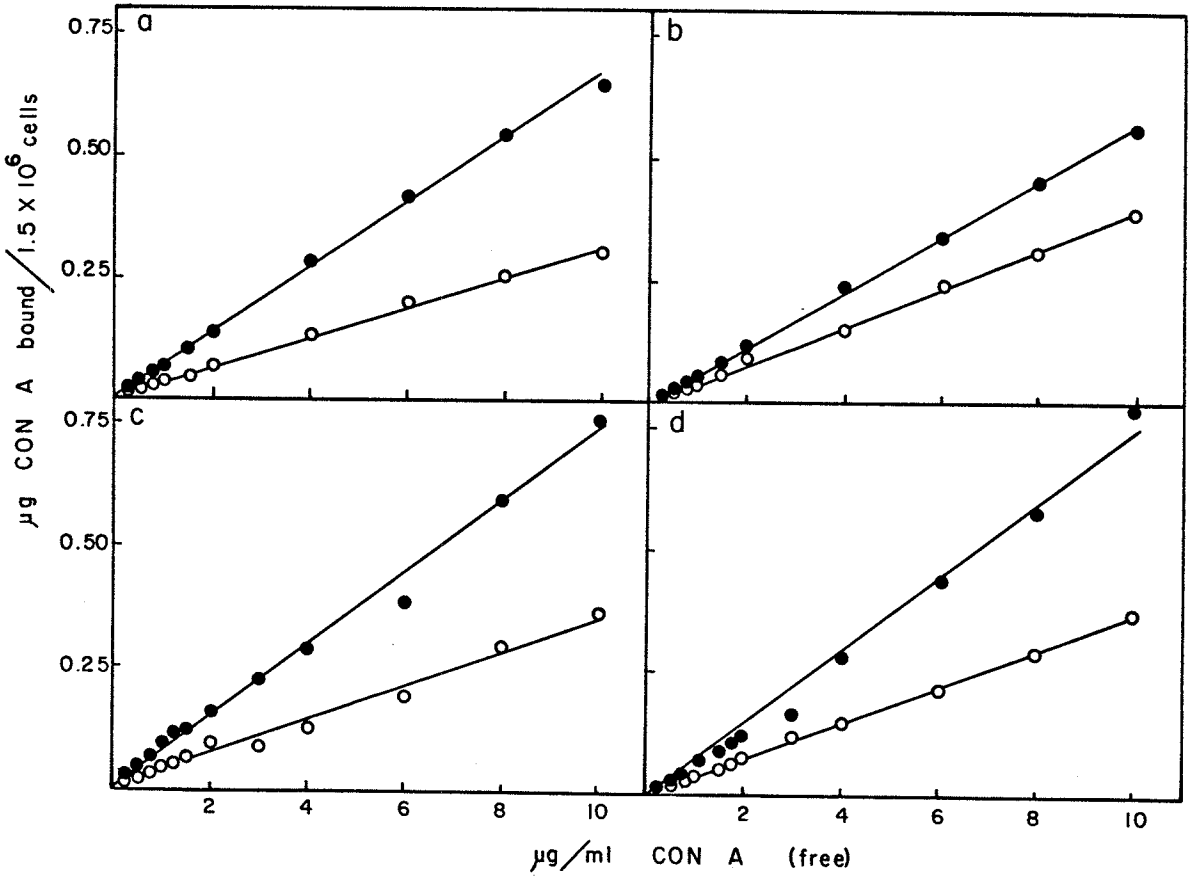
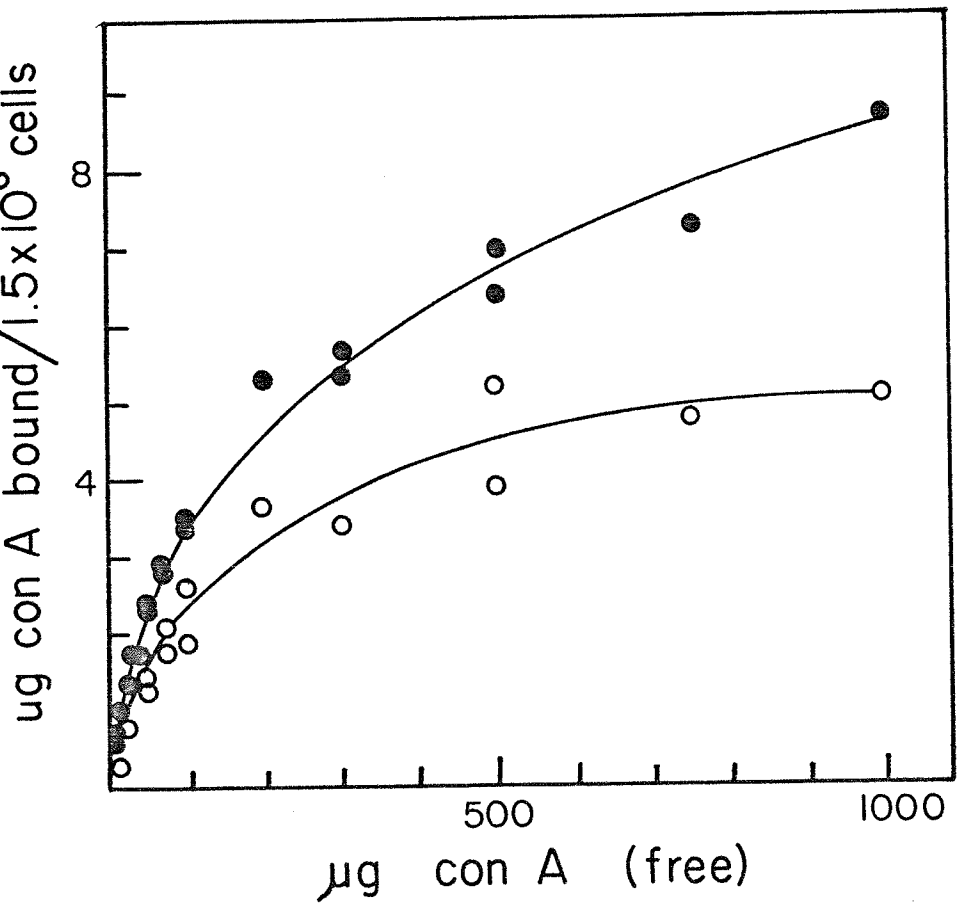

a
b
c
d
0.75
0.50
0.25
2
4
6
8
10
µg CON A bound / 1.5 X 10^6 cells
µg/ml CON A (free)

Figure 27: [^{3}H] con A binding to intact wild type and con A-resistant myoblasts at 4^{o}C between 0.25 µg/ml and 1000 µg/ml lectin. The data plotted is the sum of two independent experiments comparing binding of [^{3}H] con A to L6CL2V1, o—o, and wild type clone 2, •—•, over the range of lectin concentrations indicated. Myoblasts were grown to confluence in medium supplemented with 10% foetal bovine serum.


ug con A bound/1.5x10^6 cells
8
4
500
1000
µg con A (free)

method (Scatchard, 1949), a linear plot (Figure 28) was observed (correlation coefficient of 0.93). However when the data obtained for the wild type cells was analyzed by the Scatchard plot, the line through the set of points appeared to be a composite of two straight lines (correlation coefficient of 0.95). This suggests the presence on wild type cell surfaces of two general sets of receptors (high and low affinity sites) for con A. When the line in the Scatchard plot is extrapolated to the abscissa an estimate is obtained for the amount of lectin bound/culture at saturation binding. By this method it was estimated that wild type clone 2 cells bound 9.4 μg of con A/1.5 x 10^6 cells while L6CL2V1 cells bound only 6.1 μg/1.5 x 10^6 cells. These values represent 1.25 x 10^7 molecules/cell and 8.13 x 10^6 molecules/cell for wild type and variant lines respectively. Interestingly, binding data for Chinese hamster ovary cells showed similar values for con A bound/cell with wild type and con A-resistant lines (Wright and Ceri, 1977 a,b).

Since the selection of con A-resistant myoblast lines occurred at either 34° or 37°C (Parfett *et al.*, 1981; Figure 6, this thesis), it was of interest to determine if the differences in the amount of lectin bound to variant and wild type cells observed at 4°C could also be detected at the higher temperature. As shown in Figure 29, unlike many other cultured cell lines, the rat L6 myoblasts bound approximately the same amount of labelled con A at 37°C as was previously observed at 4°C indicating that internalization of the lectin

Figure 28: Scatchard analysis of [^{3}H] con A binding to intact wild type and con A-resistant myoblasts. The data of Figure 27 has been recalculated and presented according to Scatchard's equation (Scatchard, 1949): $B/F = nk - Bk$, where B is the amount of lectin bound, F is the free lectin concentration, n is the amount of lectin bound at saturation, and k is the apparent association constant for lectin: receptor site binding.

o——o, wild type clone 2;

Δ——Δ, L6CL2V1

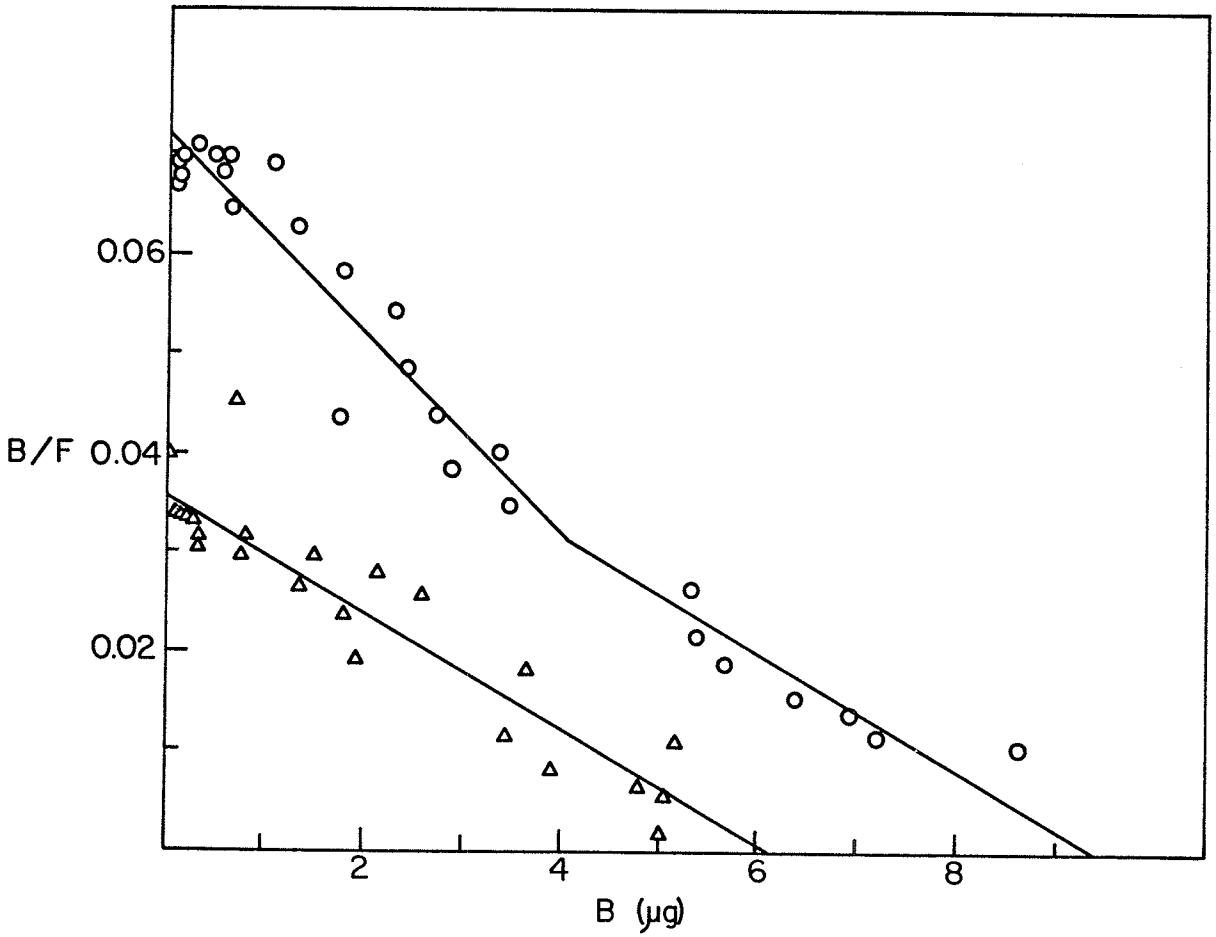
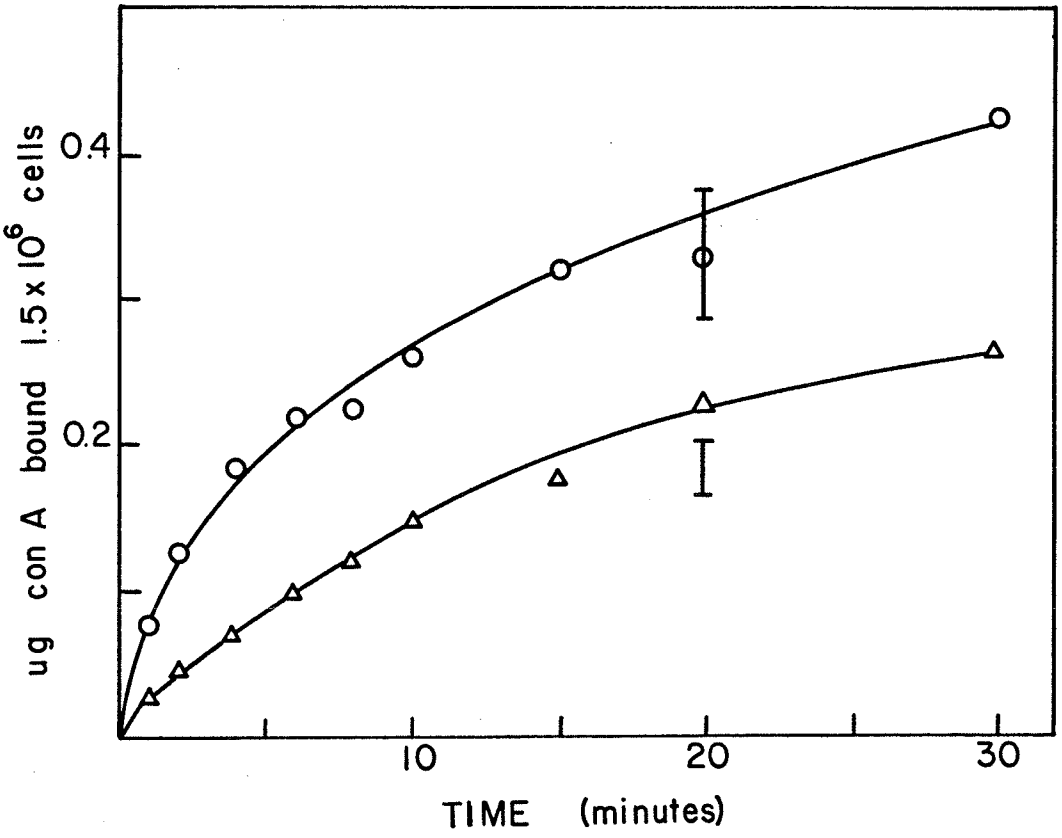

0.06
B/F 0.04
0.02
2
4
6
8
B (µg)

Figure 29: [^{3}H] con A binding to intact wild type and con A-resistant myoblasts at 37^{o}C. o——o, wild type clone 2; Δ——Δ, L6CL2V1. Confluent cells were washed with warm saline and immediately tested for [^{3}H] con A binding in saline without the metabolic inhibitor, NaN_3. For comparison, the range of [^{3}H] con A binding measured during experiments at 4^{o}C for 20 min, with 10 mM NaN_3 is indicated by the vertical bars.

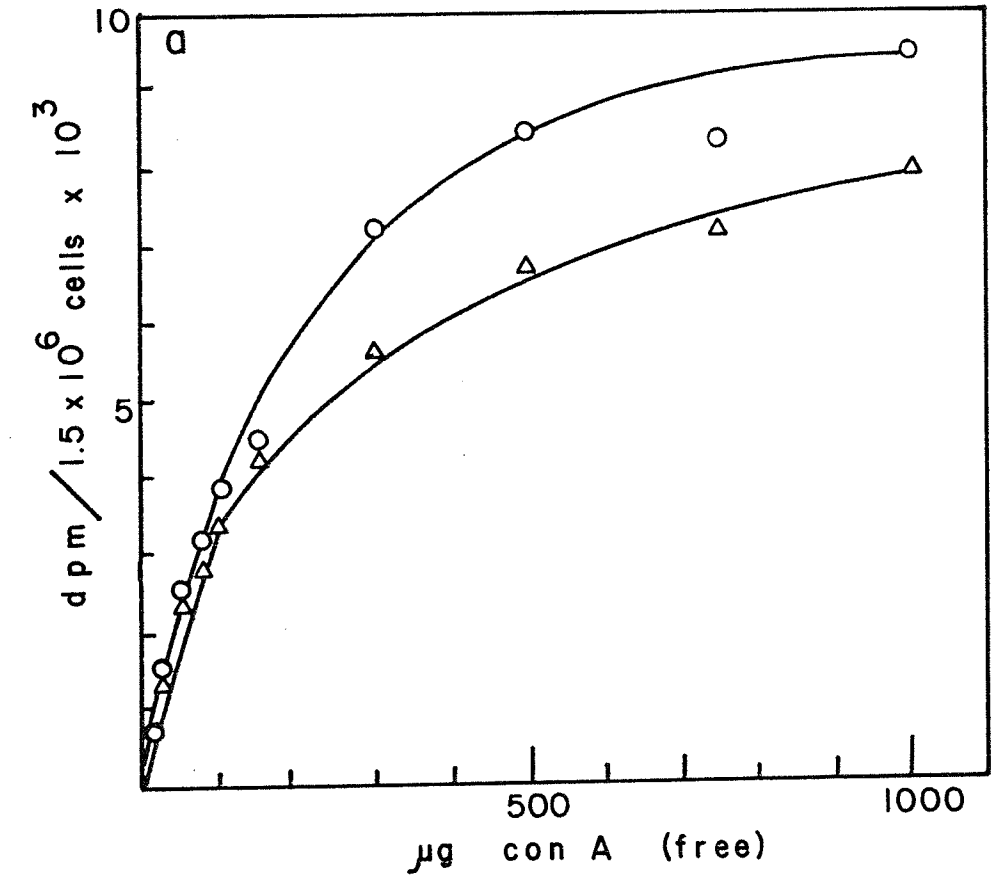

ug con A bound 1.5 x 10^6 cells
0.4
0.2
10
20
30
TIME (minutes)

is not a rapid process with L6 myoblasts at the higher temperature and apparently does not complicate interpretations of the binding data. Also a significant reduction in con A binding to variant as compared to wild type cells was observed at 37°. This is in contrast to Chinese hamster ovary cells which rapidly internalize con A at higher temperatures (Storrie, 1979; Storrie and Edelson, 1977) so that con A-resistant and wild type cells do not show the same relative difference in con A binding at room temperature which is detected at 4°C (Wright and Ceri, 1977b; Wright, 1973).

B Resistance to Con A is Associated with Specific Cell Surface Receptors

Lectin binding experiments shown in Figures 26 to 29 were performed with cells previously grown for at least three generations in medium supplemented with 10% foetal bovine serum. To determine whether the serum component in the growth medium would alter the lectin binding properties, wild type myoblasts grown in either 10% foetal bovine serum or 10% calf serum were compared for con A binding. The results shown in Figure 30 reveal that more binding sites are available at the cell surface when cells are grown in calf serum. However, when wild type and con A-resistant myoblasts were grown in calf serum, no differences between lectin binding to variant and wild type cells were detected (Figure 31a,b). Interestingly, the differences observed (Figs. 26, 27) in the binding abilities of variant and wild type myoblasts could be restored by incubating cells previously grown in

Figure 30: A comparison of [^{3}H] con A binding to wild type clone 2 myoblasts after growth in medium supplemented with foetal bovine or calf serum. o——o, calf serum; Δ——Δ foetal bovine serum. (a) Increase of binding to intact myoblasts with increasing lectin concentrations. (b) Presentation of the data in (a) according to Scatchard's equation. The cells grown in medium containing 10% foetal bovine serum were cultured for at least three generations after being harvested from medium containing calf serum. The binding experiments were performed at 4^o C in saline containing 10mM NaN_3.


a
10
5
dpm / 1.5 x 10^6 cells x 10^3
500
1000
µg con A (free)

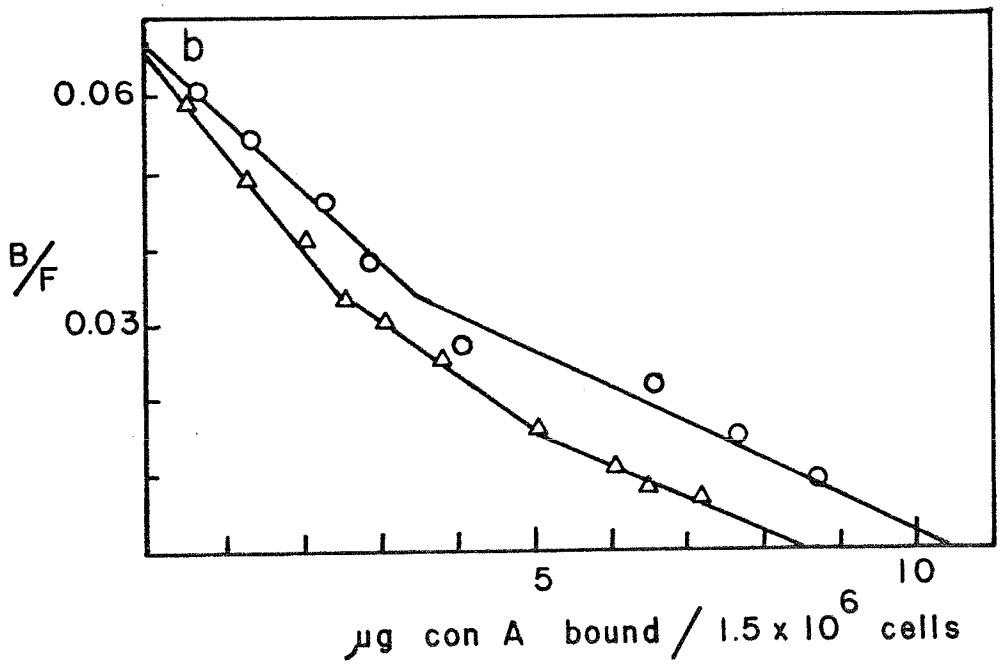
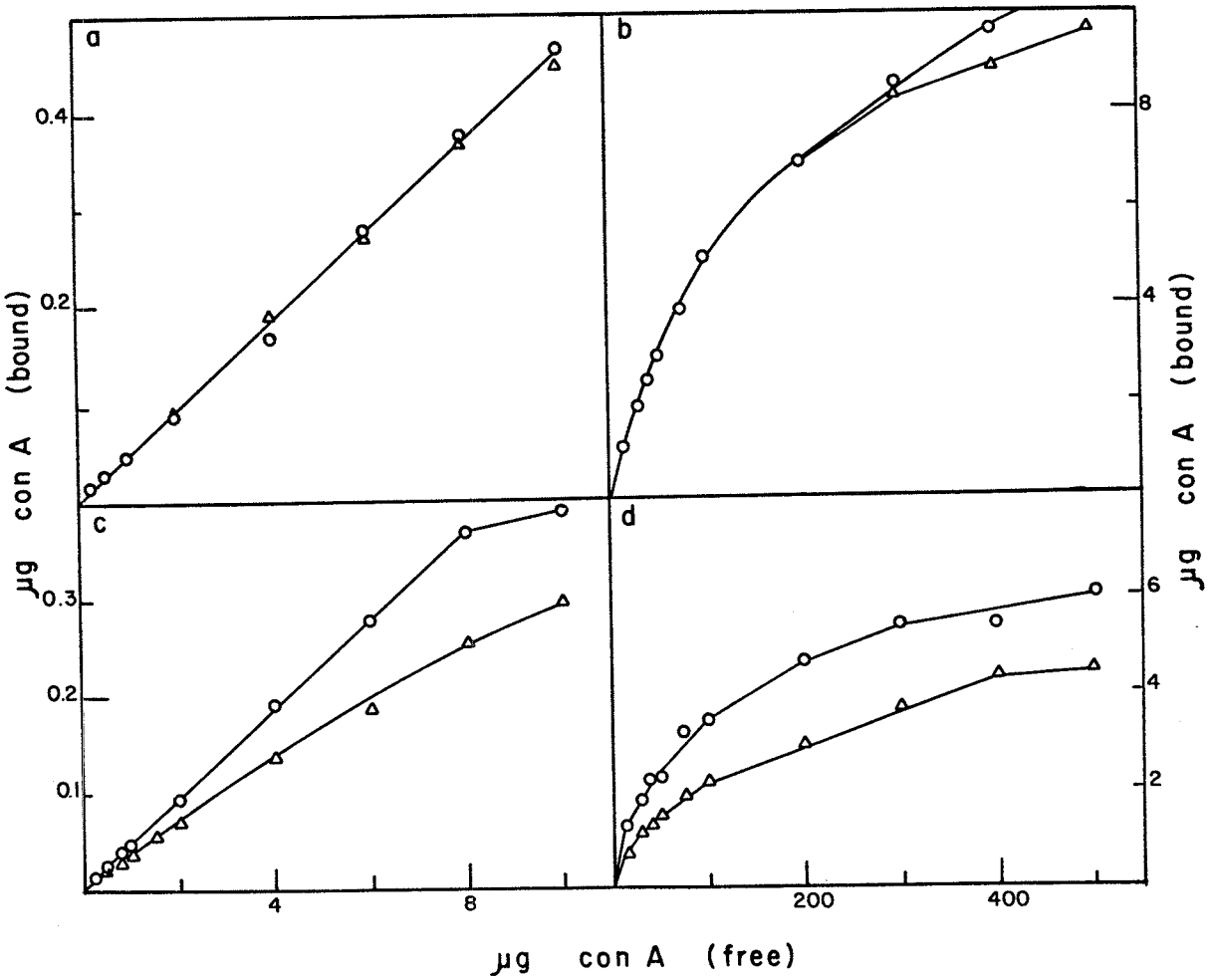

b
0.06
0.03
B/F
5
10
µg con A bound / 1.5 x 10^6 cells

Figure 31: The effect of serum on [^{3}H] con A binding to intact wild type and con A-resistant myoblasts. o——o, wild type clone 2; Δ——Δ, L6CL2V1. (a and b) cells previously grown in medium containing 10% calf serum: (c and d) cells grown in medium containing 10% calf serum, followed by 16 to 18 hours incubation in medium with no serum supplement.


a
b
c
d
0.4
0.2
0.3
0.2
0.1
8
4
6
4
2
4
8
200
400
µg con A (bound)
µg con A (bound)
µg con A (free)

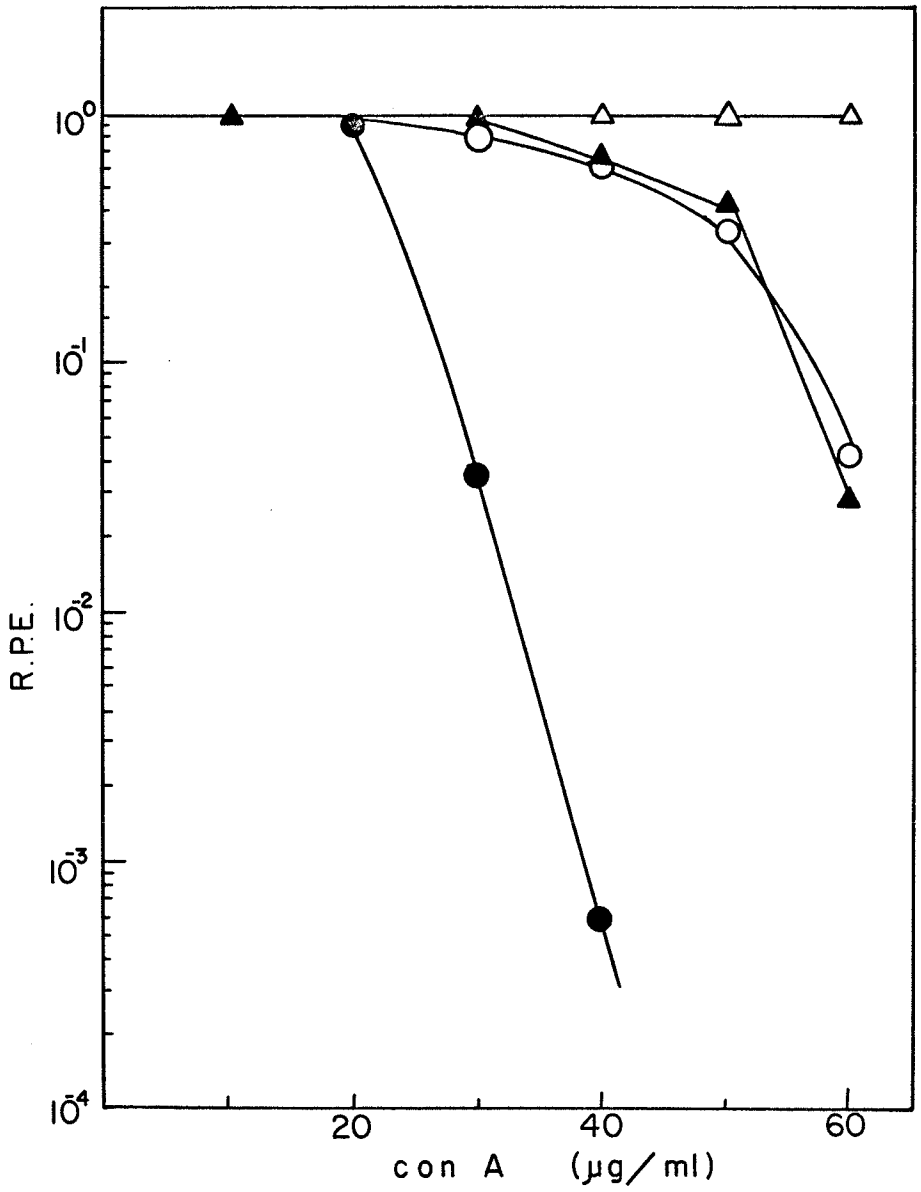
serum, in medium without serum for
18 h (Figure 31c,d). The most likely
con A binding with medium composition
ble of incorporating into cell surfaces
rotein from the calf serum. These
ptors for [^{3}H]con A. Turnover of
ccurs when myoblasts are incubated
for a period of time in the absence of calf serum.

The sensitivity of variant and wild type myoblasts to con A cytoxicity was compared with cells previously cultured in the presence of medium containing either 10% calf serum or 10% foetal calf serum (Figure 32). Although the cytotoxic effects of con A were different when cells were grown in the presence of calf as opposed to foetal calf serum, the variant line was obviously more resistant to con A that the parental wild type line in medium supplemented with either calf or foetal calf serum. Higher concentrations of lectin were needed to reduce the relative plating efficiencies of both cell populations cultured in the presence of calf serum. It is likely that con A binds to many serum glycoproteins and the final free concentration of lectin available for binding to cell surfaces is lower in medium containing calf serum as compared to foetal calf serum.

C Membrane Proteins with Altered Oligosaccharides

A reduction in the mannosylation of the lipid intermediates of glycoprotein biosynthesis was detected with the four con A-resistant myoblasts (Section V). Therefore, it was of interest to determine what

Figure 32: The effect of various concentrations of con A on the relative colony forming ability of wild type and con A-resistant myoblasts after growth in medium supplemented with either foetal bovine serum or calf serum. o——o, wild type clone 2; Δ——Δ, L6CL2V1. Solid figures, 10% foetal bovine serum; open figures, 10% calf serum.


R.P.E.
10^0
10^-1
10^-2
10^-3
10^-4
20
40
60
con A (μg/ml)

effect this alteration might have on the mannose content of membrane glycoproteins. Cytoplasmic membranes of two variant lines and their parental wild type populations were prepared after metabolic labelling in the presence of [^{3}H]mannose. The membrane proteins were solubilized and separated by SDS slab gel electrophoresis. Figure 33 shows the typical Coomassie blue stained peptide pattern; no obvious differences were observed in repeated separations. Stained gels were sliced into equal strips and the distribution of radioactivity within the tracks was examined.

The total amount of radioactivity incorporated into membrane glycoproteins was routinely greater in the variant cell line L6CL2V1 than in its wild type parental line during mannose incorporation experiments; therefore, to determine if mannose incorporation was altered as a result of increased uptake, the incorporation of [^{3}H] mannose into cells was examined as a function of time. Over the time period examined (26 minutes) the variant line took up about 35% more mannose than wild type cells. Increased mannose uptake of this nature could account for the elevated levels of label recovered from the membrane protein preparations, although other explanations are possible (e.g., see Section V, C). For this reason, the label distribution among gel slices was expressed as a percentage of total radioactivity recovered.

Comparison of the [^{3}H] label distribution in wild type and variant L6CL2V1 membrane glycoproteins revealed some important differences (Figure 35). For example, peaks with molecular weights of approximately 32,000d and 44,500d were significantly reduced in the con A-resistant

Figure 33: Coomassie blue stain of the cell surface membrane proteins separated by SDS slab gel electrophoresis. The results represent three independent experiments. Molecular weight standards include bovine haemoglobin, mw 15,000; rabbit muscle aldolase, mw 40,000; bovine serum albumin, mw 66,000; serum albumin cross-linked dimer, mw 132,000. (a) Membrane preparation from wild type clone 2 and (b) membrane preparation from L6CL2V1 myoblasts.

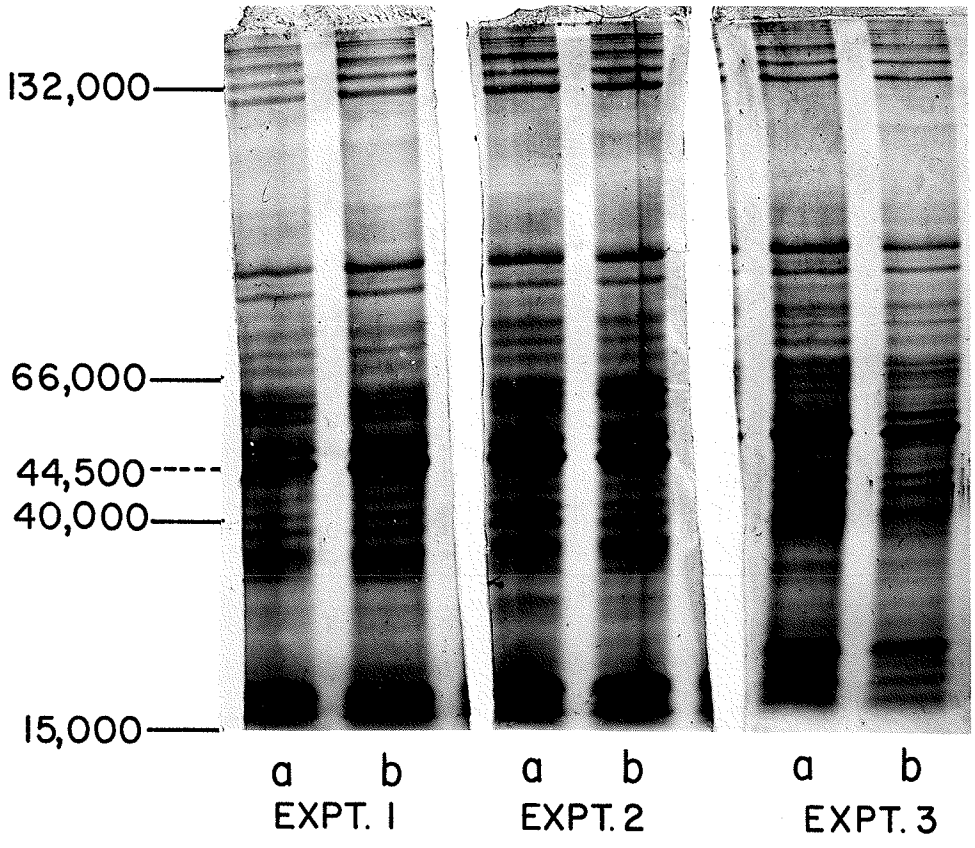

132,000
66,000
44,500
40,000
15,000
a b
EXPT. 1
a b
EXPT. 2
a b
EXPT. 3

Figure 34: Cellular uptake of [^{3}H] mannose by wild type and con A-resistant myoblasts. o——o, wild type clone 2; Δ——Δ, L6CL2V1. Uptake was measured at 37^{o}C in medium supplemented with 0.1% calf serum, 0.5 μCi of [^{3}H] mannose and 0.2 mM cold mannose.

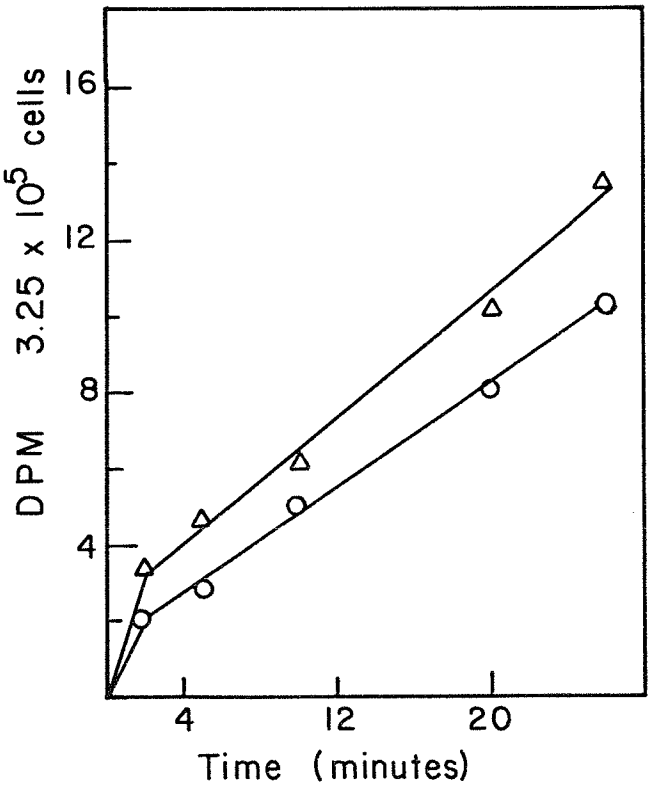
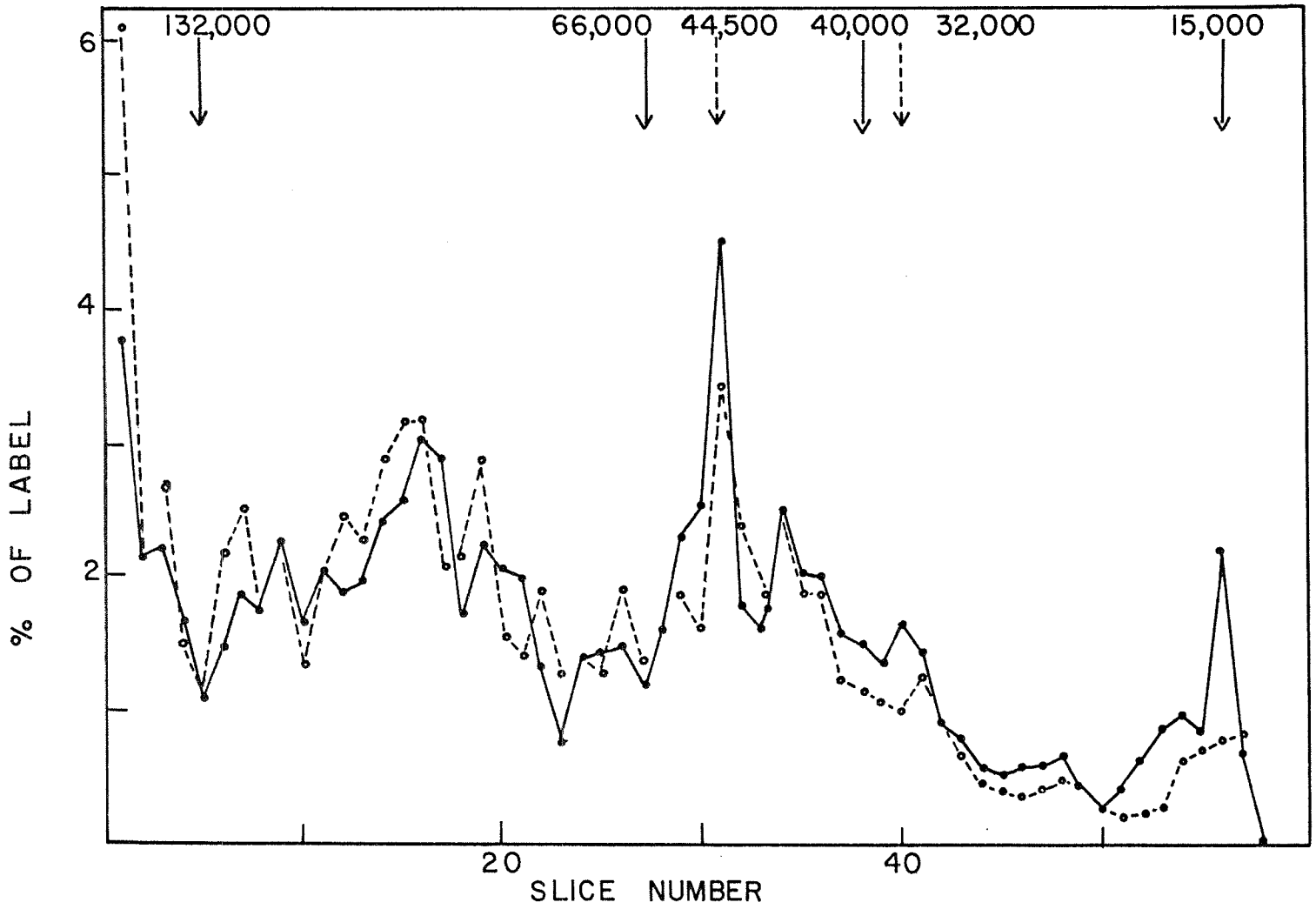

DPM 3.25 x 10^5 cells
16
12
8
4
4
12
20
Time (minutes)

Figure 35: Distribution of label into cell surface membrane preparations following [^{3}H] mannose incorporation and SDS slab gel electrophoresis. Adjacent tracks of a slab gel containing wild type and variant separations were sliced into 1.5 mm portions and the amount of radioactivity was determined in a liquid scintillation counter. The solid line indicates L6C12 and the broken line shows L6C12V1. The molecular weights indicated by the arrows represent the relative positions of bovine haemoglobin (15,000d), rabbit muscle aldolase (40,000d), bovine serum albumin (BSA) (66,000d), and BSA crosslinked dimer (132,000d).


132,000
66,000
44,500
40,000
32,000
15,000
6
4
2
% OF LABEL
20
40
SLICE NUMBER

cell populations. Also, low molecular weight material running near the dye front of the gels appeared to contain relatively less radioactivity in membrane preparations from variant cells. These differences were also observed in the independent variant line L6CL5V1 when compared to its parental wild type cells (Figure 36).

D Direct Lectin Staining of Membrane Glycoproteins Separated by SDS-PAGE

Direct application of con A to gels was used to analyse the glycoproteins of cell surface membranes according to both oligosaccharide specificity and apparent molecular weight. This technique has been very useful in the mapping of membrane glycoproteins from Chinese hamster ovary cells (Horst *et al.*, 1979) and in detecting alterations in cellular glycoproteins during events such as viral transformation (Burridge, 1976). Figure 37 shows parallel tracks from a SDS gel fractionating total cytoplasmic membrane proteins from myoblasts. The gel was equilibrated in Tris buffer and the denatured proteins within were allowed to react with [^{125}I]-labelled con A. The lectin binds seven major bands of glycoprotein in wild type clone 2 and L6CL2V1 cell surface membranes as revealed by autoradiography of the destained gel. The major band of [^{125}I] con A binding corresponded to the major peak of [^{3}H] mannose incorporation at 44,500d (see Figures 36 and 37), and was observed in both wild type and con A-resistant myoblasts. The staining intensity of wild type and variant glycoprotein appeared to be identical in the 44,500d region as well as at all other bands

Figure 36: Distribution of label into cell surface membrane preparations following [^{3}H] mannose incorporation and SDS gel electrophoresis in an independent wild type myoblast line and the con A-resistant variant selected from it. The solid line indicates wild type clone 5 and the broken line shows L6CL5V1. Molecular weight markers are as explained in Figure 35.

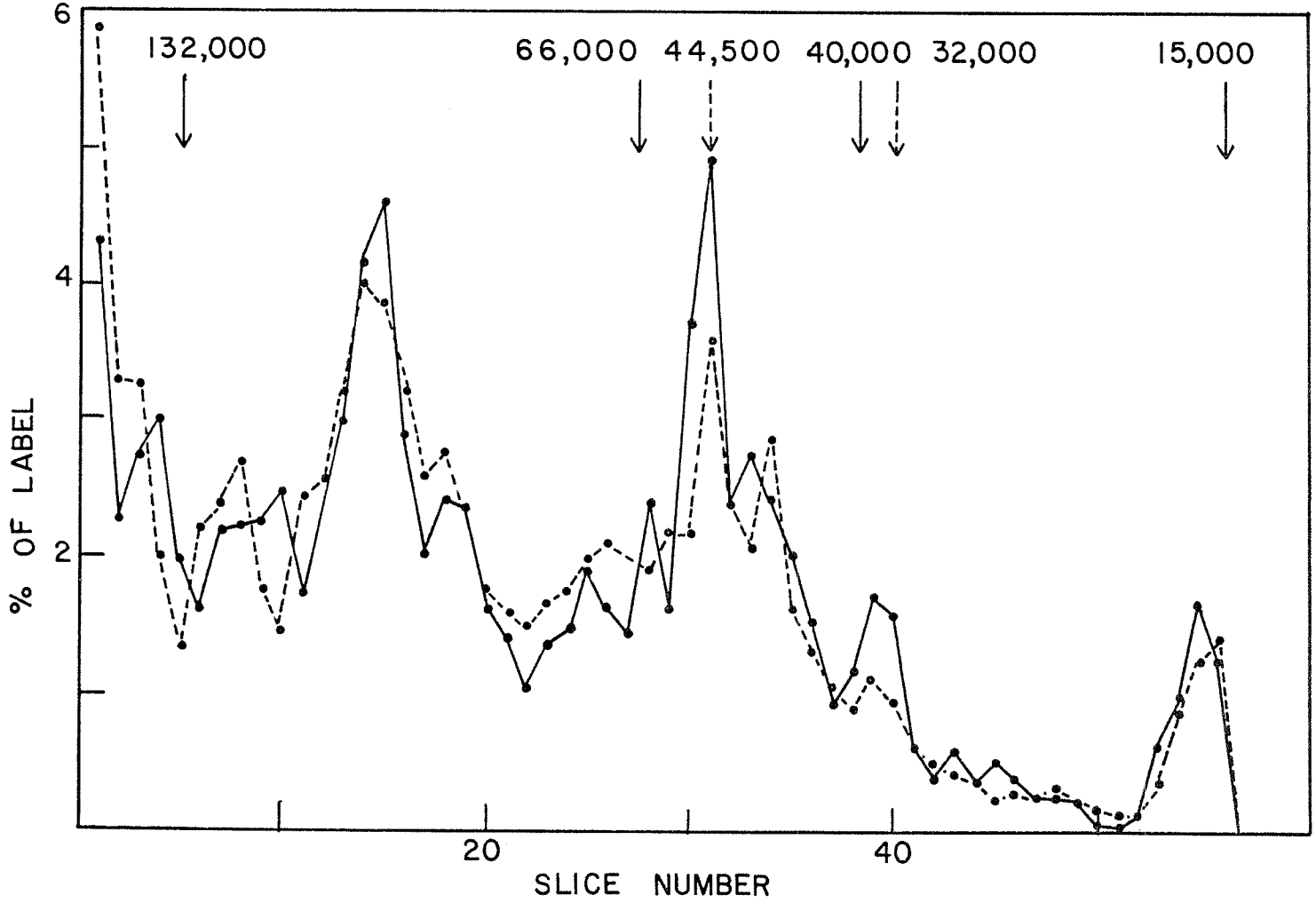
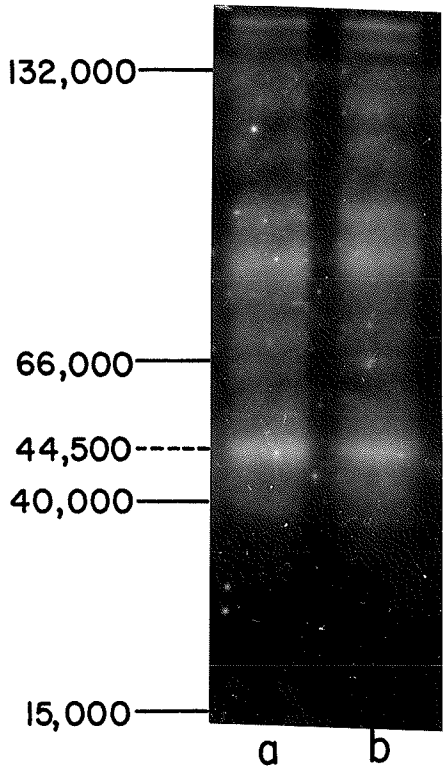

132,000
66,000
44,500
40,000
32,000
15,000
6
4
2
% OF LABEL
20
40
SLICE NUMBER

Figure 37: Direct [^{125}I] con A staining of membrane glycoproteins from wild type and con A-resistant myoblasts separated by SDS gel electrophoresis. (a) L6CL2V1. (b) wild type clone 2. See Figure 33 for the identity of molecular weight standards. After running the gel and fixing the separated proteins, the tracks were equilibrated in binding buffer. [^{125}I] con A (0.5 μCi/ml) in binding buffer was overlayered on the gel and incubated at room temperature for 24 hours, followed by destaining in excess binding buffer. Areas in which con A remained bound were visualized by autoradiography.

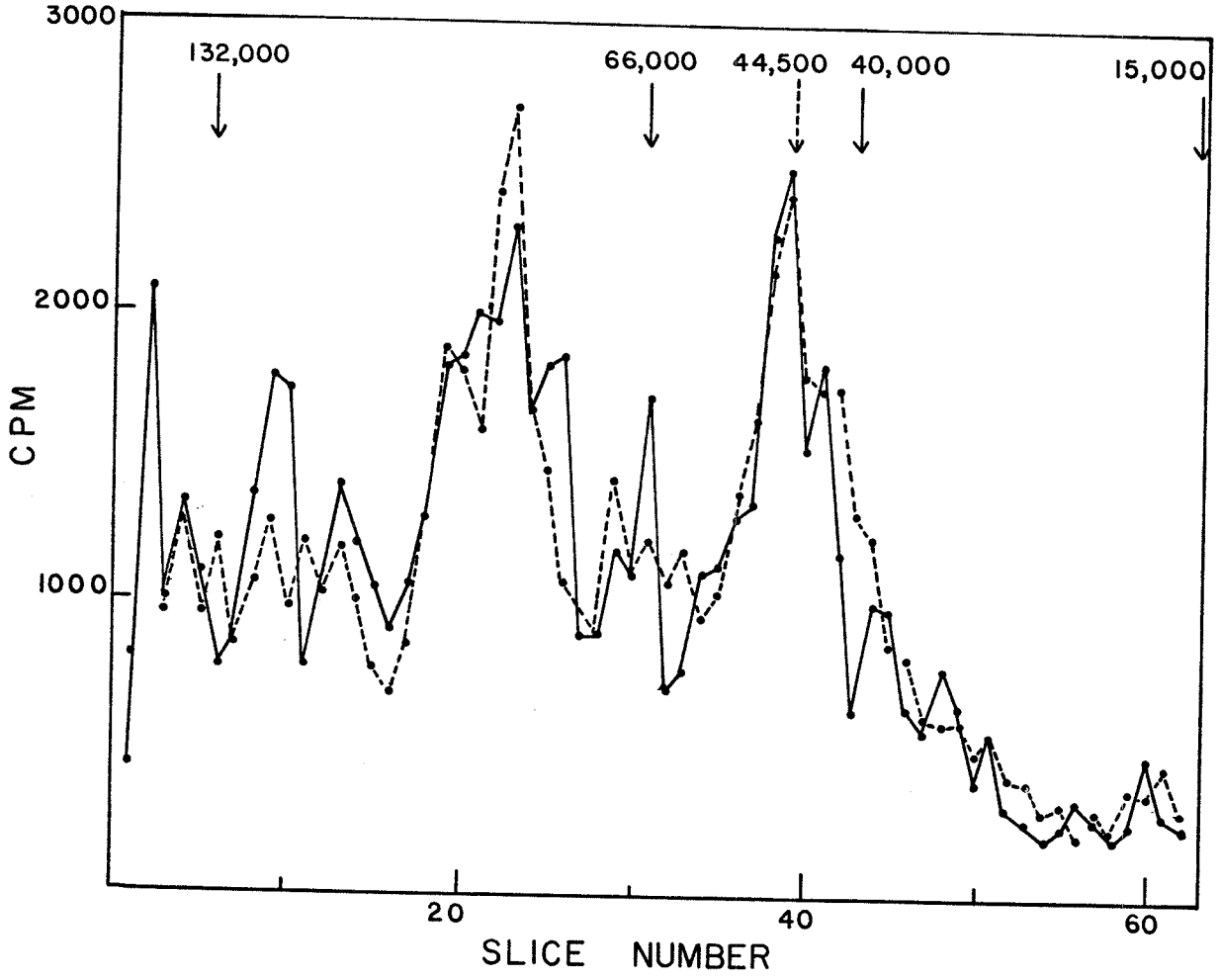

132,000
66,000
44,500
40,000
15,000
a
b

in the tracks. A more quantitative estimate was obtained by slicing the same gel and determining the amount of [^{125}I] label in each slice by scintillation counting. Again, the overall pattern of lectin staining between wild type and variant appeared to be similar. Several small differences were visible at slices 10, 26 and 43 which were not detected by autoradiography (Figure 38).

E Con A Sepharose Column Chromatography of [^{3}H] Mannose Labelled Glycoproteins

Affinity chromatography with con A covalently bound to Sepharose has proven especially useful in the fractionation of glycoproteins and glycopeptides (Lloyd, 1976). Modifications of monosaccharide composition and arrangement may either enhance or reduce the ability of a mannose containing oligosaccharide to interact with the lectin. Analysis of the elution profile of the oligosaccharide serves as a sensitive indicator of its affinity for con A (Narasimhan *et al.*, 1978). Since no differences in the total binding of con A to isolated membrane glycoproteins could be detected, the affinity of that binding was examined using the chromatographic technique. The 44,500d peak of [^{3}H] mannose incorporation was chosen for analysis because a large difference between variant and wild type had been demonstrated (see Figures 35 and 36). The labelled band was cut from the dried gel containing separations of wild type clone 2 and L6CL2V1 membrane proteins and rehydrated in 0.1 M Tris buffer, pH 8.0 containing 0.01 M $CaCl_2$ and 2 mg/ml pronase. Digestion of the protein was continued

Figure 38: Quantitative determination of [^{125}I] con A bound to the membrane proteins of wild type and con A-resistant myoblasts separated by SDS gel electrophoresis. The gel tracks shown in Figure 37 were sliced into 1.5 mm sections and the radioactivity in each slice was determined by scintillation counting. Solid line indicates wild type clone 2; broken line indicates L6CL2V1.


3000
2000
1000
CPM
132,000
66,000
44,500
40,000
15,000
20
40
60
SLICE NUMBER

for 48 hours at 37°C. More than 95% of the radioactivity was released from the gel and was applied to a con A sepharose column to be eluted with a linear gradient of α-methyl-D-mannoside. Similar amounts of radioactivity were bound to the Sepharose relative to material eluting with buffer for both wild type and variant preparations (85% and 83% respectively). The patterns of elution of bound glycopeptides were different (Figure 39). About 30% of the wild type radioactivity was released before reaching a concentration of 0.05 M α-methyl-D-mannoside whereas 90% of the variant glycopeptides were released within this concentration range. Higher sugar concentrations were required to remove about 70% and 10% of the remaining radioactivity from wild type and variant preparations respectively.

The experiment was repeated with wild type clone 5 and L6CL5V1 membrane glycoprotein within the 44,500d region, but using glycopeptides extracted from three separate tracks of the gel for each cell line (Figure 40). The elution profile was essentially identical to that shown for clone 2 wild type and L6CL2V1 glycopeptides. Approximately 50% and 70% of the digested glycopeptides bound to the column (variant and wild type respectively). Elution of the bound fraction was 95% complete at 0.05 M α-methyl mannoside for variant glycopeptides, while only 31% of wild type label had been eluted at this point in the gradient.

The 44,500d band in all of these cell lines probably contains a mixture of glycopeptides with varying affinities for con A. There appears to be a substantial shift to glycopeptides in the variant

Figure 39: Con A sepharose affinity chromatography of pronase digest of the 44,500d [^{3}H] mannose labelled glycoprotein band. The solid line indicates label recovered from a wild type digest (clone 2) and the broken line shows label recovered from a variant (L6CL2V1) digest. The diagonal broken line indicates the α-methyl mannoside gradient. Total radioactivity is from a single area (44,500d) within the track of separated membrane proteins from each cell line.

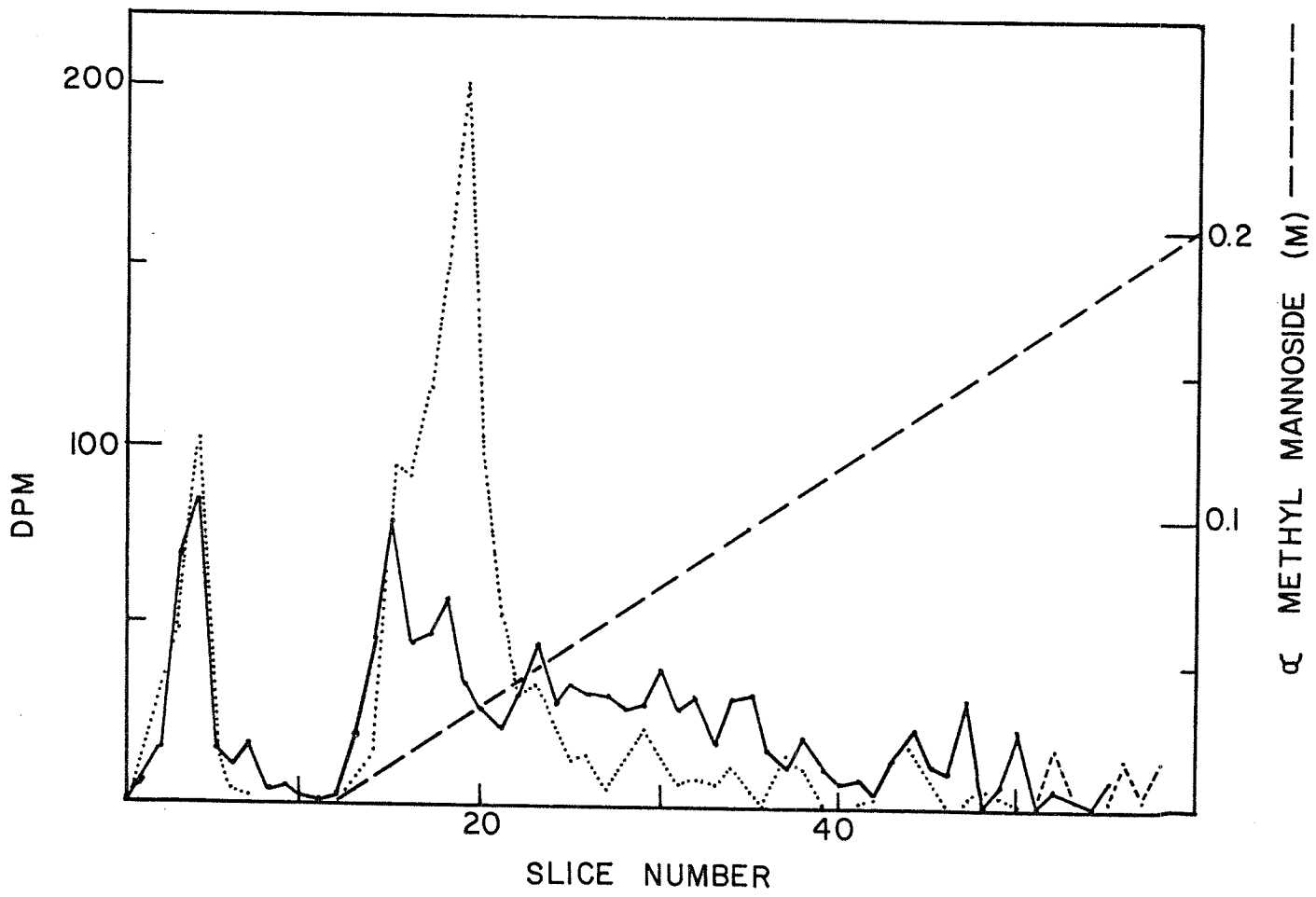
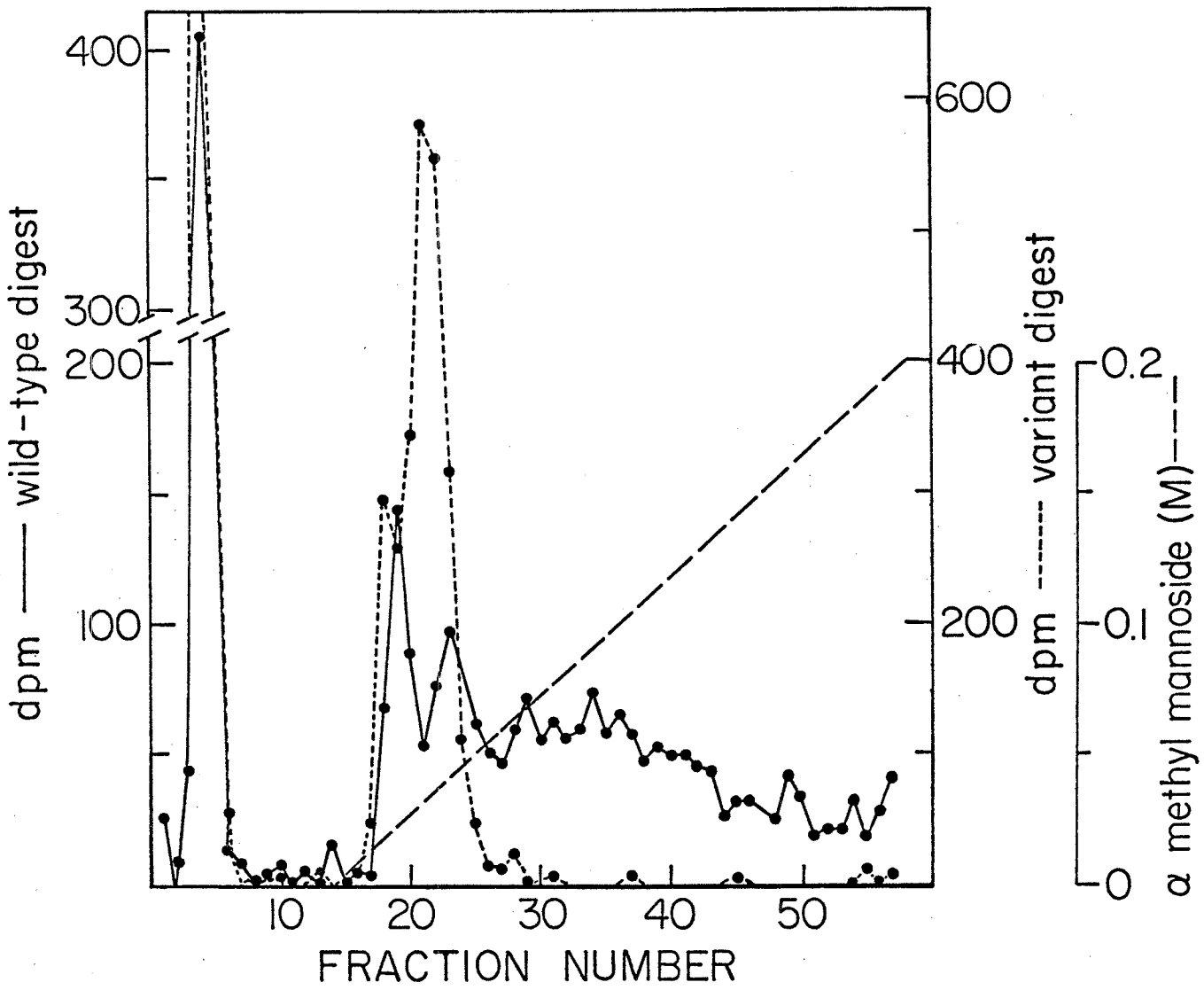

200
100
DPM
20
40
SLICE NUMBER
0.2
0.1
α METHYL MANNOSIDE (M)

Figure 40: Con A sepharose affinity chromatography of a pronase digest of the 44,500d [^{3}H] mannose labelled glycoprotein band of an independent wild type myoblast line and the con A-resistant variant selected from it. The solid line indicates label recovered from a digest of wild type clone 5 and the broken line shows label recovered from L6CL5V1. The diagonal line indicates the α-methyl mannoside gradient. Total radioactivity is from a single area (44,500d) within 3 separate SDS-PAGE tracks of separated membrane proteins from each cell line.


400
300
200
100
dpm —— wild-type digest
600
400
200
dpm ----- variant digest
0.2
0.1
0
α methyl mannoside (M)
10
20
30
40
50
FRACTION NUMBER

cell populations which have lower affinities for con A. These results support the [^{3}H] con A binding studies with intact cells (Figures 26 to 28) which indicated a reduction in high affinity sites on con A-resistant myoblasts.

Section V Altered Glycoprotein Biosynthesis Activity of Wild Type and Con A-resistant Myoblasts

A Mannose Transferase Activity of Wild Type and Con A-resistant Myoblasts

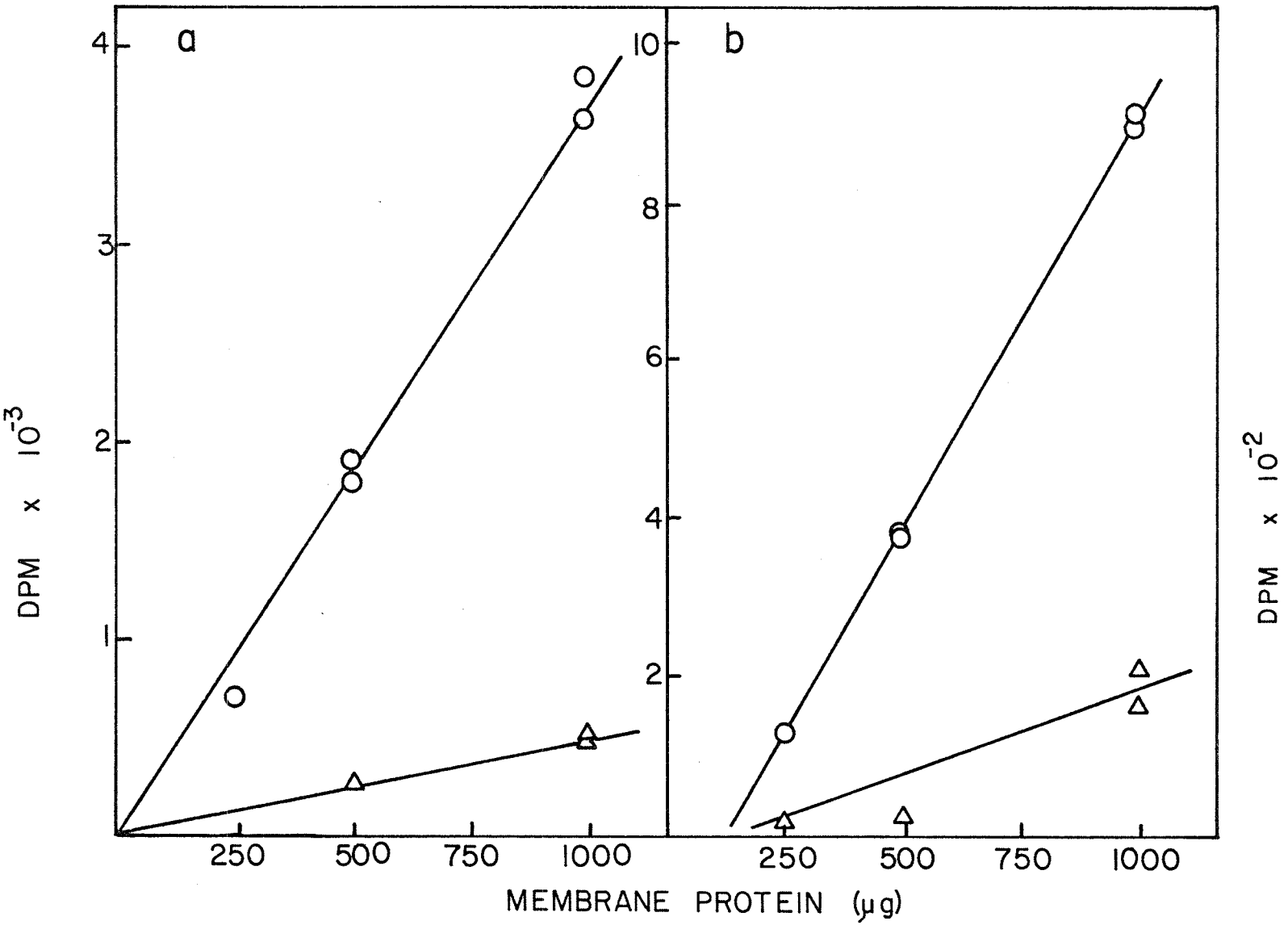
A biosynthetic scheme as complicated as that which is currently being described for the synthesis of glycoprotein oligosaccharides contains numerous possible enzymatic steps in which a genetic defect might give rise to an alteration of the final product (see History section: Structure and Biosynthesis of Membrane Glycoproteins). Structurally altered oligosaccharides resulting from such lesions are likely to have lectin affinities which differ significantly from those of normal cells. Previous studies using con A-resistant Chinese hamster ovary cells (Wright *et al.*, 1979, 1980; Krag, 1979), and mouse lymphoma Cells (Chapman *et al.*, 1980) have shown that the lesions recovered by lectin selection are restricted to the pathway leading to synthesis of the lipid intermediates. The result is a reduction in the normal mannose content of cellular glycoproteins. Tenner and Scheffler (1979) described a conditionally lethal mutant

cell line which at elevated temperature does not transfer any carbohydrate from the lipid intermediate to proteins which normally have N-glycosidically linked oligosaccharide groups. Presumably, the lack of any carbohydrate on newly synthesized glycoproteins is a lethal condition and this type of mutant would not normally be recovered in a lectin selection.

Therefore, the mannosyl transferase activities leading to synthesis of lipid intermediates were studied as a possible cause for the synthesis of defective glycoproteins at the con A-resistant myoblast cell surface.

A microsomal membrane fraction was prepared from L6CL2V1 and wild type clone 2 cells to examine its ability to catalyse lipid-linked mannose and lipid-linked oligosaccharide formation from GDP-[^{14}C]-mannose (Figure 41) Under the conditions of assay employed in this study, the membranes from both wild type and variant cells incorporate radioactivity into these lipid fractions. Also, incorporation was linear with increasing membrane protein. However, it is clear from the kinetics that incorporation of label into these fractions was much lower with L6CL2V1 membrane preparations. For example, one milligram of membrane protein from wild type clone 2 incorporated about 9.5 and 5 times more label into lipid and oligosaccharide-lipid than the same concentration of membrane from the con A-resistant variant. These observations, which indicate a defect in the ability to mannosylate the lipid intermediates of glycoprotein biosynthesis, are further supported by the observation that membrane preparations from the other independently selected

Figure 41: Effect of membrane protein on incorporation of label from GDP-[^{14}C] mannose into endogenous lipid and oligosaccharide-lipid fractions. (a) Lipid fractions soluble in $CHCl_3$:CH_3OH (2:1); (b) lipid-oligosaccharide fractions soluble in $CHCl_3$:CH_3OH:H_2O (10:10:3). o——o, wild type clone 2; Δ——Δ, L6CL2V1.


a
b
DPM x 10^{-3}
DPM x 10^{-2}
MEMBRANE PROTEIN (μg)
250
500
750
1000

variants also show an impairment in the transfer of label into lipid fractions when compared with parental wild type preparations (Figure 42; Parfett *et al.*, 1981).

Three wild type lines were compared with their respective con A-resistant variants for the ability to synthesize [^{14}C]-labelled oligosaccharide lipid (Figure 43). Again, all of the variants synthesize much less of this intermediate.

The serum composition apparently has a modifying influence on the activity of this portion of the pathway in myoblasts. Those lines grown in αMEM supplemented with 10% calf serum had less activity measurable in the isolated microsomes than was detected in the clone 2 wild type and variant cells which were cultured in αMEM supplemented with 10% foetal bovine serum. The reduction was more than four-fold for the wild type cells, while no activity could be detected in the variant cells after growth in 10% calf serum. A mechanism by which this regulation of oligosaccharide biosynthesis might occur has not yet been described; however, the increased number of membrane con A binding sites (see Section IV) detected when wild type and variant myoblasts are cultured in medium supplemented with calf serum may in some way be involved.

Although repeated attempts were made, no measurable amounts of label could be found in protein fractions of the microsomal membranes during incubation with GDP-[^{14}C] mannose. It is likely that transfer of mannose from lipid fractions can occur in the

Figure 42: Incorporation of label from GDP-[^{14}C] mannose into endogenous lipid soluble in $CHCl_3$:CH_3OH (2:1) by parental wild type and con A-resistant variant membrane preparations. (a) Wild type clone 2 and L6CL2V1; (b) wild type clone 5 and L6CL5V1; (c) wild type clone 6 and L6CL6V6; (d) wild type clone 1 and L6CL1V20. o——o, parental wild type lines, Δ——Δ, con A-resistant variant lines.

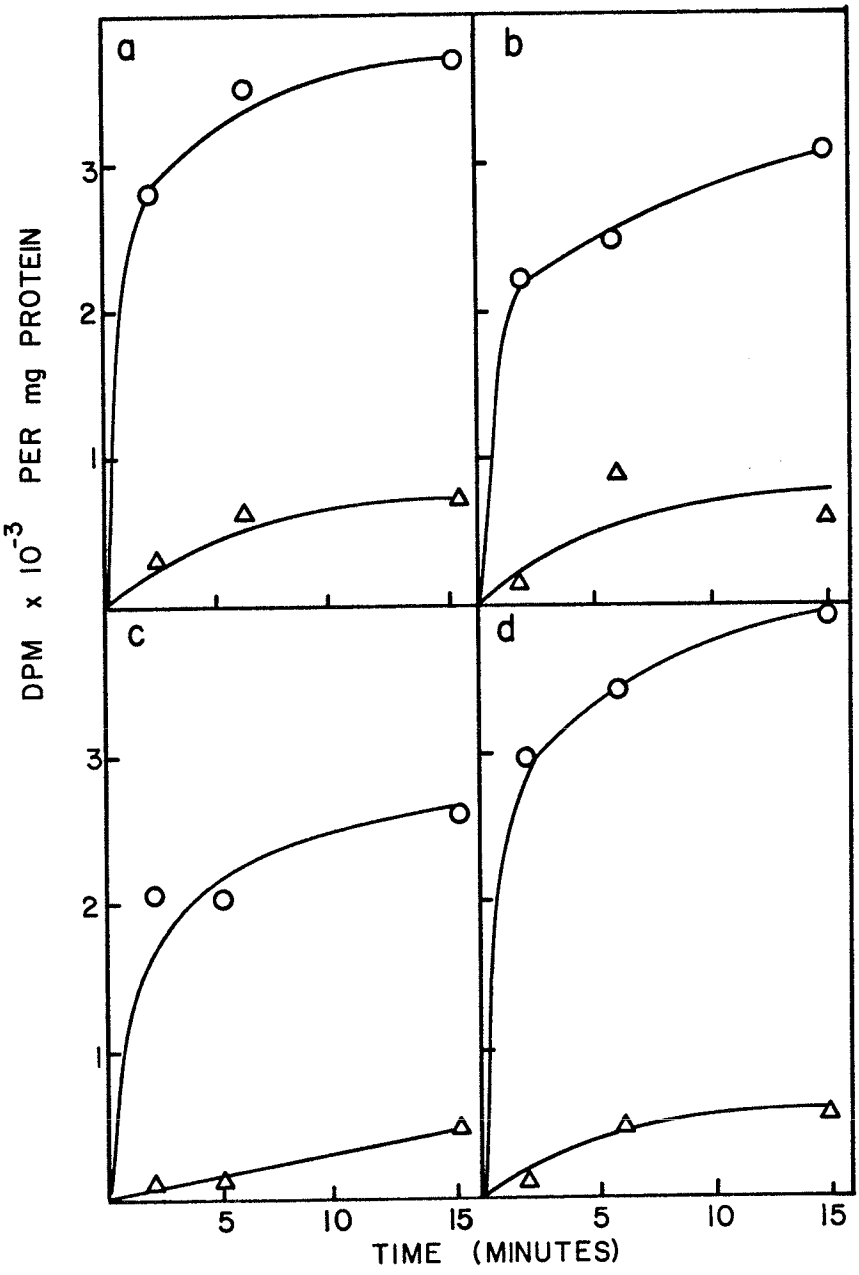
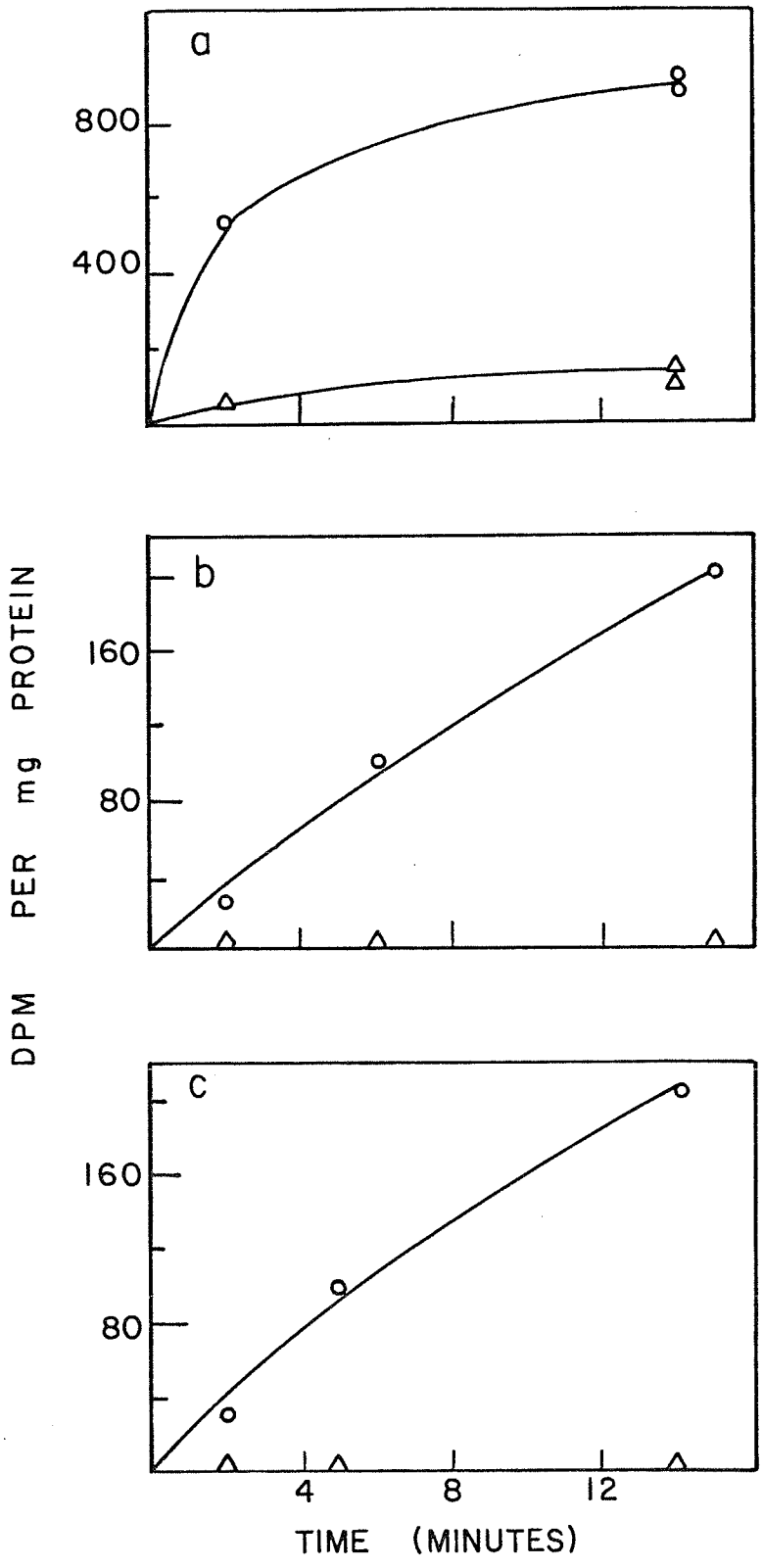

a
b
c
d
DPM x 10^{-3} PER mg PROTEIN
1
2
3
5
10
15
TIME (MINUTES)

Figure 43: Incorporation of label from GDP-[^{14}C] mannose into endogenous lipid-oligosaccharide soluble in $CHCl_3$: CH_3OH:H_2O (10:10:3) by parental wild type and con A-resistant variant membrane preparations. (a) Wild type clone 2 and L6CL2V1. (b) Wild type clone 5 and L6CL5V1. (c) Wild type clone 6 and L6CL6V6. o——o, parental wild type lines; Δ——Δ, con A-resistant variant lines. Wild type clone 2 and L6CL2V1 were previously grown in 10% foetal bovine serum whereas clone 5 and clone 6, wild type and variant lines were cultured in medium supplemented with 10% calf serum.


a
800
400
b
160
80
c
160
80
4
8
12
TIME (MINUTES)
DPM PER mg PROTEIN

isolated vesicles, but that the conditions provided by the assay procedure are not suitable for this portion of the pathway. It was obvious that alternate conditions would be required to examine mannosylation of protein in this cell line. Previously, a permeabilized cell assay had been developed in this laboratory to measure the activity of the intracellular enzyme ribonucleotide reductase (Lewis *et al.*, 1978; Wright *et al.*, 1981). Treatment of whole cells with the detergent Tween-80 leaves the cytoplasmic membrane in a state which allows small molecules to freely pass into the cytoplasm. The macromolecular components with the cell membrane remain intact to the extent that enzyme activities such as DNA synthesis may be measured (Billen and Olson, 1978).

When GDP-[^{14}C] mannose was added to permeabilized cells and incubated at 37^{o}C, radioactivity became associated with lipid and oligosaccharide-lipid fractions. After complete extraction of these fractions, a significant amount of label was recovered in the protein pellet (Table IX). It is likely that the entire, lipid-linked mannosyl transferase pathway is operating under these conditions and that the mannose remaining with the protein pellet is covalently attached to intracellular acceptor proteins. The lipid-oligosaccharide fraction which becomes radioactive is significantly larger after labelling by this procedure than was previously measured by incubating isolated microsomal membranes with GDP-[^{14}C] mannose. For example, at 20 minutes of incubation, permeabilized whole wild type cells contain 19% of the label recovered in lipid fractions as oligo-saccharide-lipid, while isolated membranes have only 5 to 7% as

Table IX. Tween 80-Permeabilized L6 Myoblasts as the Source of Enzyme in the Mannosyl Transferase Assay.

	Time (min)	clone 2 wild type	L6CL2V1
mannolipid	20	23,385*	7,427
$CHCl_3$:CH_3OH	40	17,493	10,129
2:1	60	-	4,876
lipid oligosaccharide	20	5,312	4,035
$CHCl_3$:CH_3OH:H_2O	40	6,238	2,993
10:10:3	60	7,571	2,384
protein	20	3,843	5,324
	40	9,710	7,430
	60	7,242	5,712

*All activity is expressed as dpm per 10^7 permeabilized cells. Each assay tube contained 100 nCi ($\simeq$ 200,000 dpm) of GDP-[^{14}C] mannose. The results are corrected for background by subtraction of the radioactivity measured in an assay stopped at 0 time.

Similar results were observed in two independent experiments.

oligosaccharide-lipid after growth in calf serum (see Figures 41 and 42).

A relative reduction in lipid intermediate synthesis is readily apparent in permeabilized L6CL2V1 myoblasts when compared with wild type clone 2 myoblasts. Also, the radioactivity associated with protein is reduced in the variant cells at later times.

B Formation of Dolichol-P-mannose From Exogenous Dolichol-phosphate

Previous studies have shown that con A-resistant cell lines are defective in the ability to incorporate label from GDP-[^{14}C] mannose into the lipid intermediates needed for synthesizing asparagine-linked oligosaccharides (Wright *et al.*, 1980; Wright, 1979). In Chinese hamster ovary cells, this lesion appears to be due to a lack of free dolichol required for the transfer of the last five mannose residues to the lipid-oligosaccharide structure involved in protein glycosylation (Wright *et al.*, 1979; Krag, 1979). Therefore, con A-resistant membrane preparations incorporated approximately wild type levels of GDP-[^{14}C] mannose into lipid intermediates when exogenous dolichol-PO_4 was supplied (Krag, 1979). However, in con A-resistant mouse lymphoma cells the defect is in the lack of ability to synthesize dolichol-PO_4-mannose; therefore, the addition of exogenous dolichol-PO_4 did not significantly increase the incorporation of labelled mannose into lipid with membrane preparations from these variant cells (Chapman *et al.*, 1980). To determine whether the lesion in the myoblast variants resembles either the hamster or the mouse lymphoma defects, similar experiments were performed with resistant and wild type

myoblasts. Earlier studies have shown that con A-resistant myoblasts contain a defect in the ability to mannosylate lipid intermediates (Wright *et al.*, 1980; Parfett *et al.*, 1981). Table X indicates that this defect is not alleviated by addition of exogenous dolichol-PO_4. For example, in the absence of exogenous dolichol-PO_4, wild type membranes incorporated 4 to 5 times more GDP-[^{14}C]-mannose than variant preparations, and in the presence of saturating concentrations of the lipid this difference in incorporation increased further to about 50 to 55 times more label incorporated by wild type membranes. Addition of the detergent Triton X-100 to the assay system (Fig.44)to solubilize the exogenous dolichol had no inhibitory effect on the activity of the mannosyl transferase. These results closely resemble those obtained with lectin resistant mouse lymphoma cells (Chapman *et al.*, 1980), suggesting that the con A-resistant myoblasts are unable to transfer mannose from GDP-mannose to dolichol-PO_4 due to a defect directly in a mannose transferase activity.

C Identification of Radioactive Sugars Incorporated in Protein

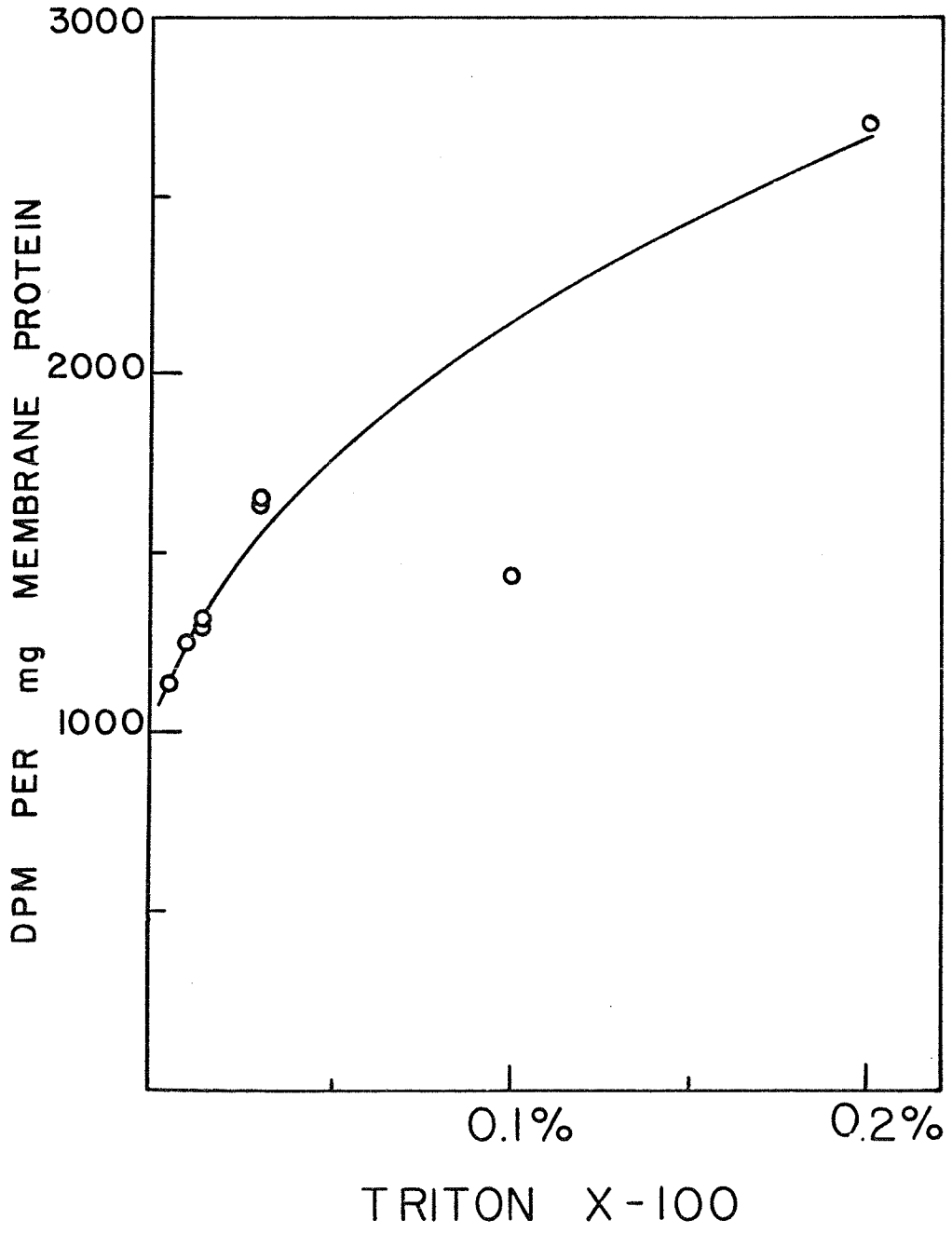
Previous studies in this laboratory (Wright *et al.*, 1979) have shown that conversion of mannose label to fucose occurs in cultured cells and that this pathway is of greater importance in con A-resistant hamster cells than in parental wild type cells. To investigate the possibility of similar changes occurring in the sugar composition of glycoproteins of lectin resistant myoblasts, wild type and L6CL2V1 cells were labelled *in vivo* with [^{14}C] mannose for eight hours and labelled membrane glycoproteins were recovered from whole cells (see

Table X. GDP-[^{14}C]mannose Incorporation[a] into Lipid with Membrane Preparations from Variant and Wild Type Myoblasts.

Cell line	Dolichol - PO_4 (μg) 0	1	2.5	10	15
L6CL2	727	975	1606	17,249	16,556
L6CL2V1	154	147	185	289	292

[a] dpm/mg membrane protein

Figure 44: Effect of various concentrations of Triton X-100 on incorporation of label from GDP-[^{14}C] mannose into endogenous lipid of wild type cells. The amount of label was reduced from the 100 nCi normally used in mannosyl transferase assays to 25 nCi.


3000
2000
1000
DPM PER mg MEMBRANE PROTEIN
0.1%
0.2%
TRITON X-100

Materials and Methods). After acid hydrolysis, mannose and fucose were separated by paper chromatography and the distribution of label in mannose and fucose is shown in Table XI. Note that radioactivity was recovered mainly in mannose and fucose with lesser amounts of label spread between the origin and mannose. Interestingly the amount of label in the mannose peak of the con A-resistant variant, L6CL2V1 was only 75% of the amount recovered from wild type clone 2 glycoproteins. However, the amount of radioactivity found in fucose in glycoprotein from variant membranes was 160% of the amount detected in the membranes of wild type cells. Therefore these results obtained with con A-resistant myoblasts (Parfett *et al.*, 1981) resemble the findings previously observed with con A-resistant Chinese hamster ovary cells (Wright *et al.*, 1979).

D HMG-Co A Reductase Activity in Wild Type and Con A-resistant Myoblasts

Many enzymes are common to the pathways of cholesterol and dolichol biosynthesis. In particular, HMG-Co A reductase which is a major regulatory enzyme for cholesterol biosynthesis (Rodwell *et al.*, 1976), can also function in the biosynthesis of isoprene units utilized for dolichol synthesis. In various cell types, the pathway to dolichol utilizes HMG-Co A reductase products at a rate which is 0.03 to 0.11% of the pathway to cholesterol (James and Kandutsch, 1979). However, suppression of HMG-Co A reductase activity by 25-hydroxycholesterol leads to concomitant inhibition of dolichyl-saccharide synthesis and dolichol-mediated glycoprotein assembly (Mills and Adamany, 1978).

Table XI. Distribution of Radioactivity Following Incorporation of [^{14}C] Mannose into Glycoprotein.

Cell Line	Radioactivity recovered, %[a]									
	Origin		Mannose		Fucose		Intermediate Region		Total	
Experiment	1	2	1	2	1	2	1	2	1	2
W.T. clone 2	5	2	39	41	18	23	19	18	81	84
L6CL2V1	3	2	29	32	34	33	20	18	86	85

[a]Radioactivity recovered is expressed as a percentage of the radioactivity in the sample applied to the paper. Results are from two experiments.

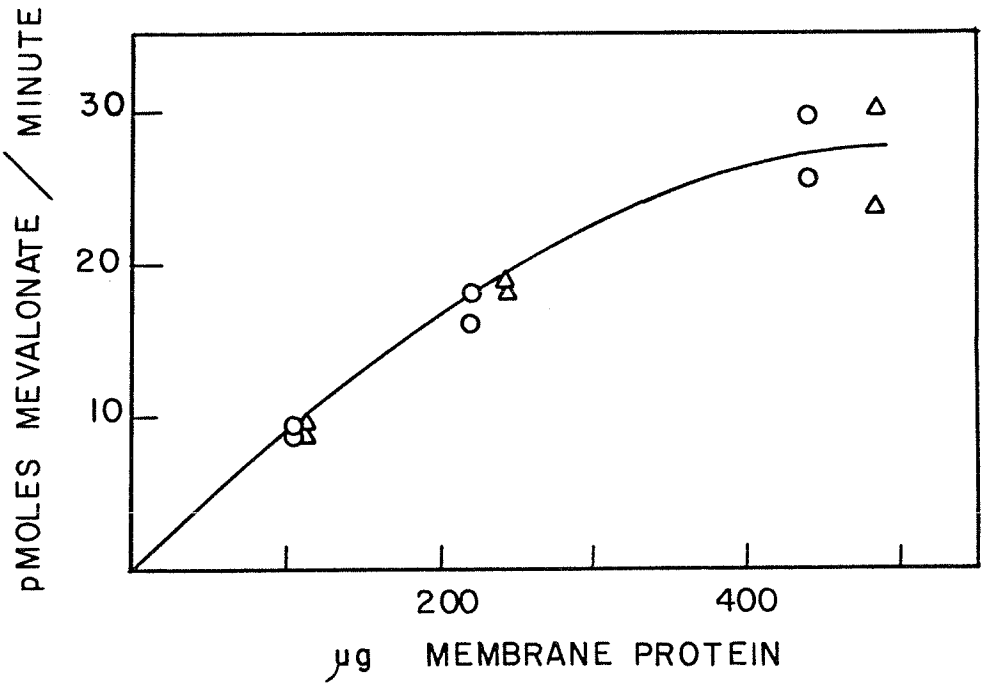
Previous results have shown that con A-resistant Chinese hamster ovary cells have unusually high levels of HMG-Co A reductase activity and that this activity responds abnormally to cholesterol supplied in the growth medium, probably because of a defective low-density lipoprotein receptor glycoprotein on the cell surface (Borgford and Burton, 1982). Therefore, the activity of the enzyme was examined in a con A-resistant myoblast line to determine if similar alterations had occurred.

Membrane preparations from both wild type clone 2 and L6CL2V1 myoblasts synthesize [^{3}H] mevalonic acid from [^{3}H] 3-hydroxy-3-methylglutaryl coenzyme A, and this activity increases with the amount of membrane protein added to the assay mixture (Figure 45). The level of activity appeared to be identical in each of these cell lines.

Section VI Con A Binding to Skin Fibroblasts from Patients with Duchenne Muscular Dystrophy and Normal Age-Matched Controls

The muscular dystrophies can be defined as disorders manifested by symptoms of abnormal muscle function in which there is no clinical, histologic or electrophoretic evidence of denervation or emotional disorder and progressive muscle weakness is evident (Rowland, 1972). Although the muscular dystrophies are hereditable, the fundamental biochemical alteration ultimately responsible for the disease is not

Figure 45: HMG-CoA reductase activity in membranes prepared from wild type and con A-resistant myoblasts. o——o, wild type clone 2; Δ——Δ, L6CL2V1.


pMOLES MEVALONATE / MINUTE
30
20
10
200
400
µg MEMBRANE PROTEIN

known. Many of the theories which have been advanced to account for the complex phenotype exhibited by human muscular dystrophies may be put into one of three categories (Rowland, 1976): the vascular theory, the neurogenic theory and the cell membrane theory. Although the first two theories are still being tested, evidence is accumulating rapidly to suggest the view that the cellular membrane is directly involved in determining the biochemical abnormalities associated with various human muscular disorders (for reviews see Lucy, 1980; Witkowski and Jones, 1981). When the membranes of erythrocytes obtained from patients with Duchenne dystrophy are examined it is obvious that the erythrocyte membrane is significantly altered. For example, reports of decreased surface membrane capping by antibodies and con A (Pickard *et al.*, 1978), stimulation of Na^+/K^+-ATPase activity by oubain - normally a potent inhibitor (Rowland, 1976), alterations in sodium and potassium fluxes (Anaki and Mawatari, 1971), a different phospholipid composition (Rowland, 1976) and abnormal membrane protein kinases (Roses *et al.*, 1975) all have indicated that the genetic change which produces Duchenne dystrophy is accompanied by biochemical alterations in the membrane of more than one cell type. Recent evidence provides support to the hypothesis that Duchenne dystrophy is expressed in cultured skin fibroblasts and can be studied in these cells (Rosenmann *et al.*, 1982).

For these reasons, the binding of [^{125}I] con A to human diploid skin fibroblasts in culture was examined to determine if any

alterations could be detected in the surface membrane architecture of cells taken from Duchenne patients. All Duchenne fibroblasts were paired with age and sex-matched cells from normal children before being tested for lectin binding. Confluent 60 mm culture dishes were incubated 24 hours in αMEM with no serum supplement prior to each experiment to eliminate possible artifacts of con A binding due to adsorbed serum proteins at the cell surface. This treatment was previously shown (Figure 31) to be effective for restoring the relative reduction in con A binding to con A-resistant L6 myoblasts after growth in calf serum - a growth condition under which they bound con A in a manner equivalent to wild type myoblasts (see Section IV). Figure 46 shows that con A binding to human fibroblast surfaces increases with the number of cells added to the culture dish (measured as protein) A significant increase in the slope of the line was observed at low cell densities; consequently, culture dishes containing less than 150 µg of protein were not used for binding experiments.

Binding of [^{125}I] con A to age-matched pairs of normal and dystrophic fibroblasts is shown in Figures 47 to 51. Saturation was usually reached by 100 µg/ml of con A. The younger patients (5 to 6 years, Figures 48 and 49) showed slightly more binding at all concentrations than was evident in the control cultures of this study. However, the fibroblast monolayers from older patients bound as much or less than their normal counterparts. Scatchard analysis (Figures 47b-51b) of the binding data from the range of lectin

Figure 46: Effect of increasing cellular protein on the binding of [^{125}I] con A to intact human diploid skin fibroblasts at 4^{o}C in saline containing 10 mM NaN_3. Various numbers of cells were plated in 60 mm culture dishes, incubated for 48 hours in the medium with 10% foetal bovine serum, then 24 hours in medium with no serum supplement before beginning the binding assay.

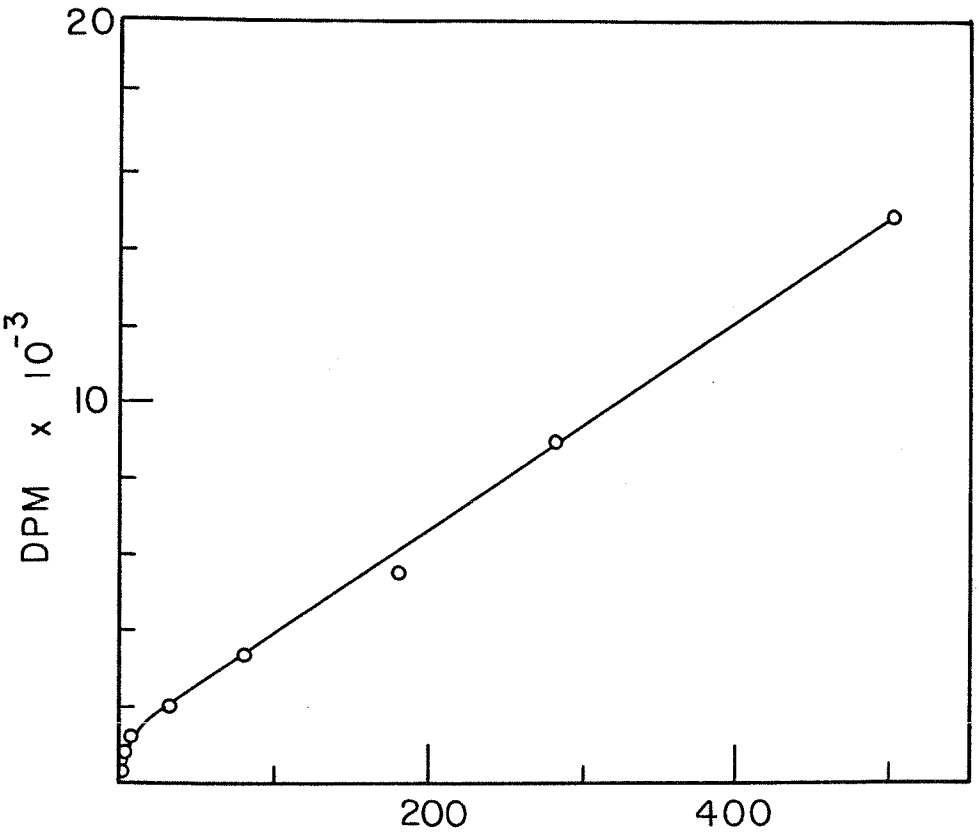
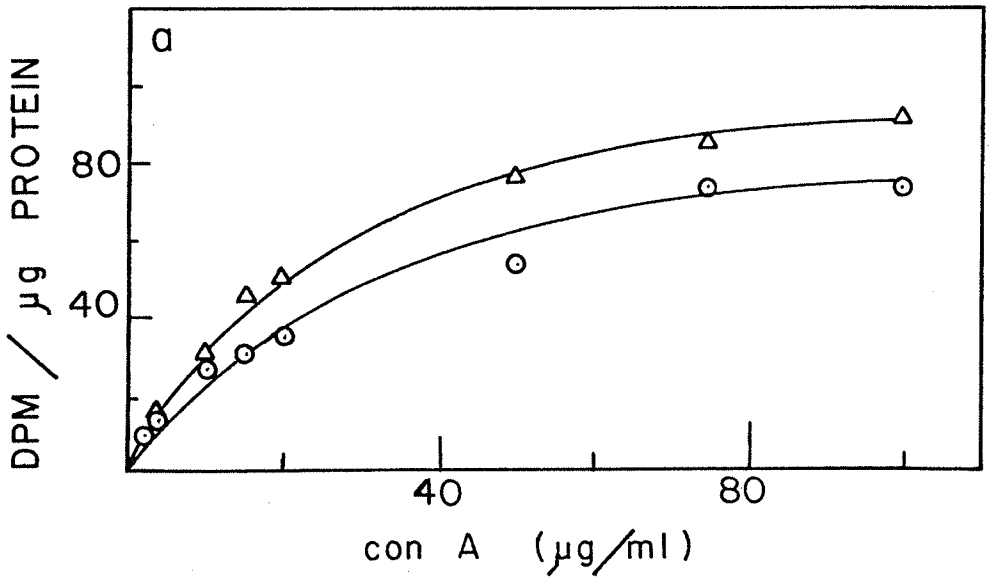

20
10
DPM x 10^{-3}
200
400
µg OF PROTEIN PER 60mm DISH

Figure 47: Effect of various concentrations of [^{125}I] con A on binding to skin fibroblasts from a 5.5 year old Duchenne muscular dystrophy patient and a 6 year old normal child. o——o, MCH 50, normal 6 year old male; Δ——Δ, WG840, Duchenne dystrophic, 5.5 year old male. (b) is a Scatchard analysis of the data presented in (a).


a
DPM / µg PROTEIN
80
40
40
80
con A (µg/ml)

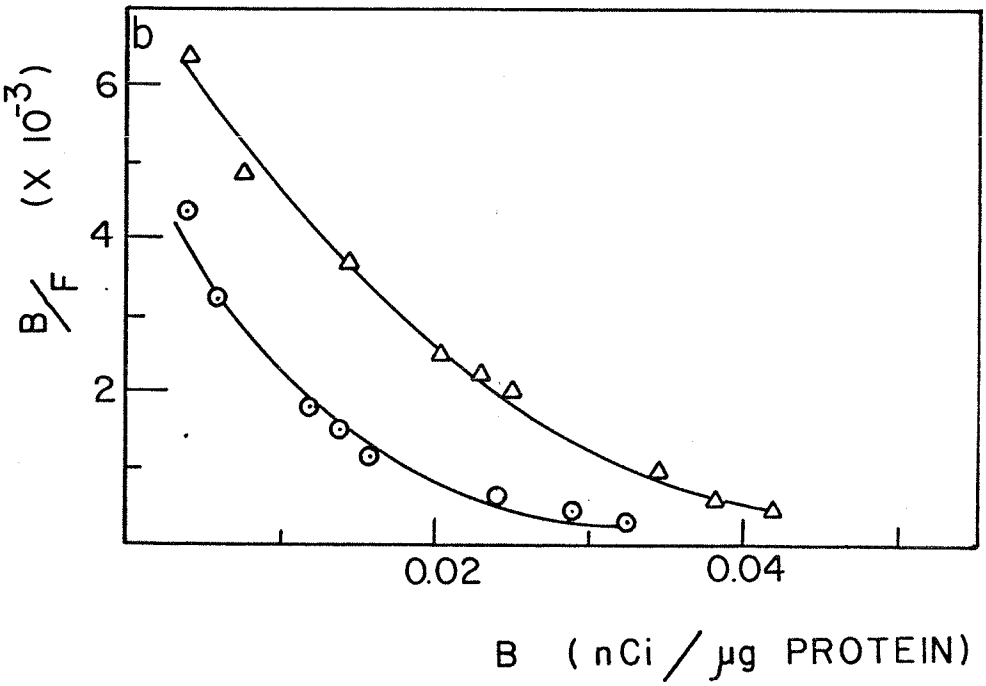
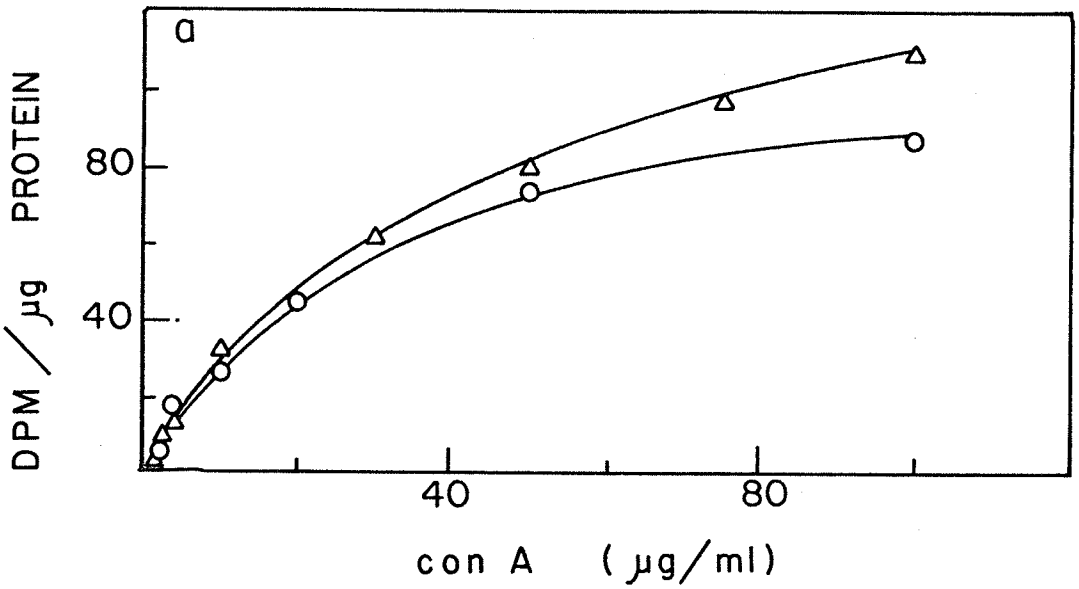

b
B/F (X 10⁻³)
6
4
2
0.02
0.04
B (nCi / µg PROTEIN)

Figure 48: Effect of various concentrations of [^{125}I] con A on binding to skin fibroblasts from a 6 year old Duchenne patient and a 7 year old normal child. o——o, MCH 52, normal 7 year old male; Δ——Δ, WG 448, Duchenne dystrophic 6 year old male. (b) A Scatchard analysis of the data presented in (a).


a
DPM / µg PROTEIN
80
40
40
80
con A (µg/ml)

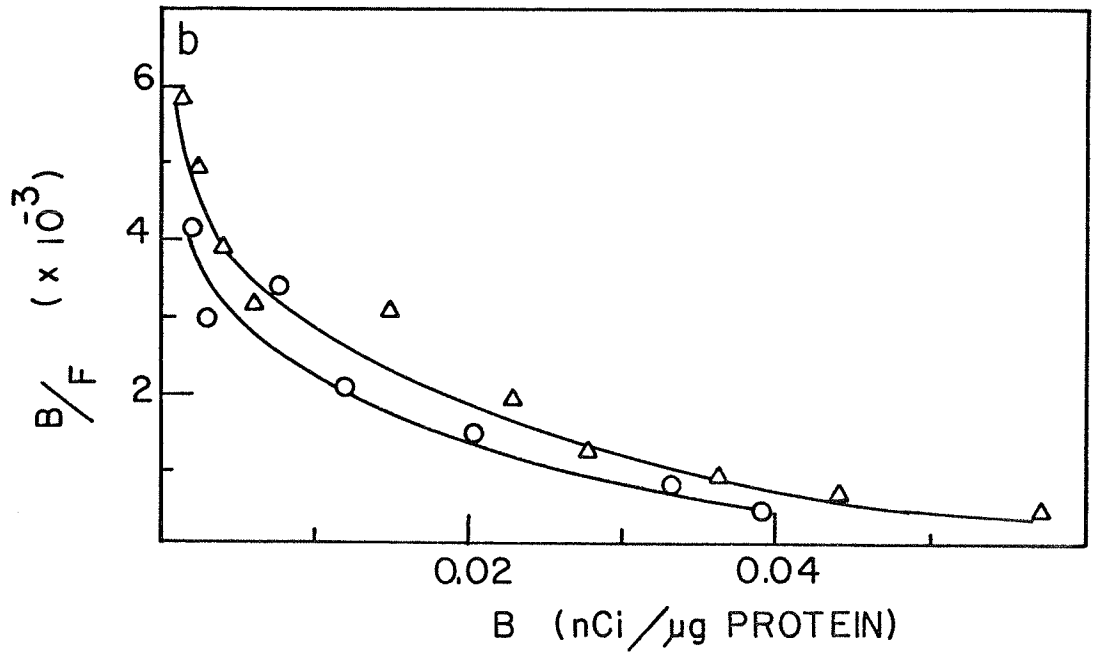
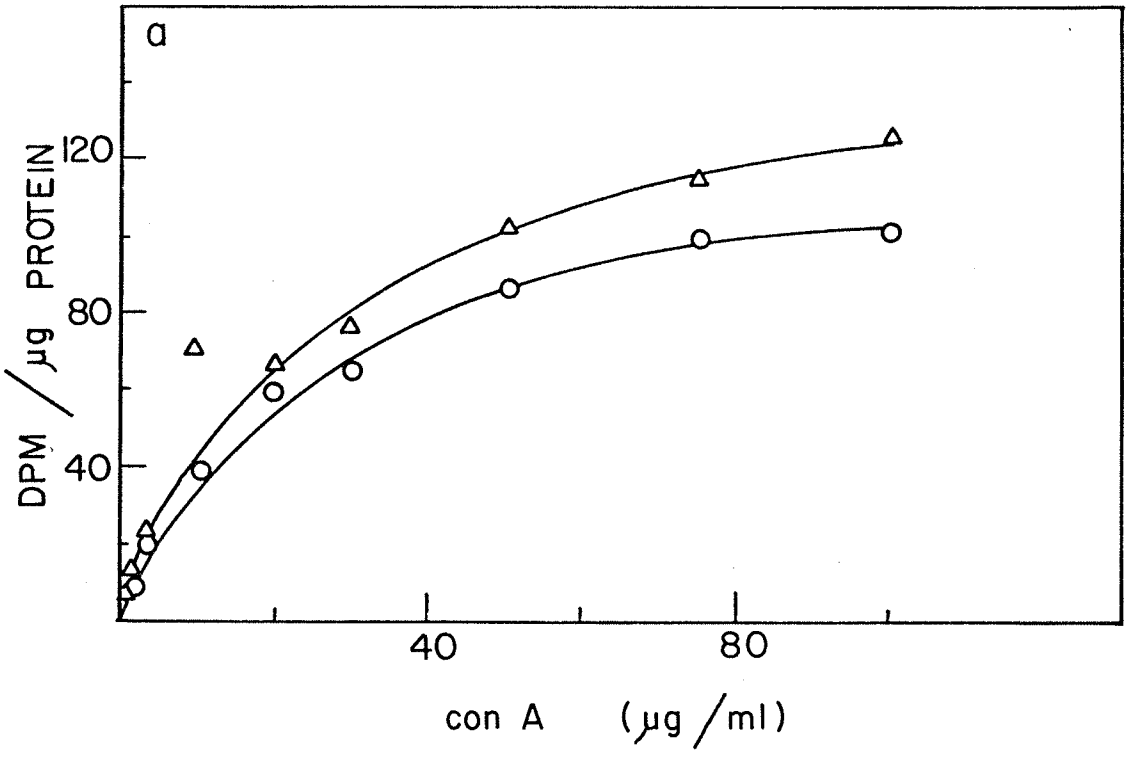

b
B/F (x 10^-3)
6
4
2
0.02
0.04
B (nCi / µg PROTEIN)

Figure 49: Effect of various concentrations of [^{125}I] con A on binding to skin fibroblasts from an 11 year old Duchenne patient and an 11 year old normal child. o——o, GM 323, normal 11 year old male; Δ——Δ, GM 3781, Duchenne dystrophic 11 year old male. (b) is a Scatchard analysis of the data presented in (a).


a
DPM / µg PROTEIN
120
80
40
40
80
con A (µg / ml)

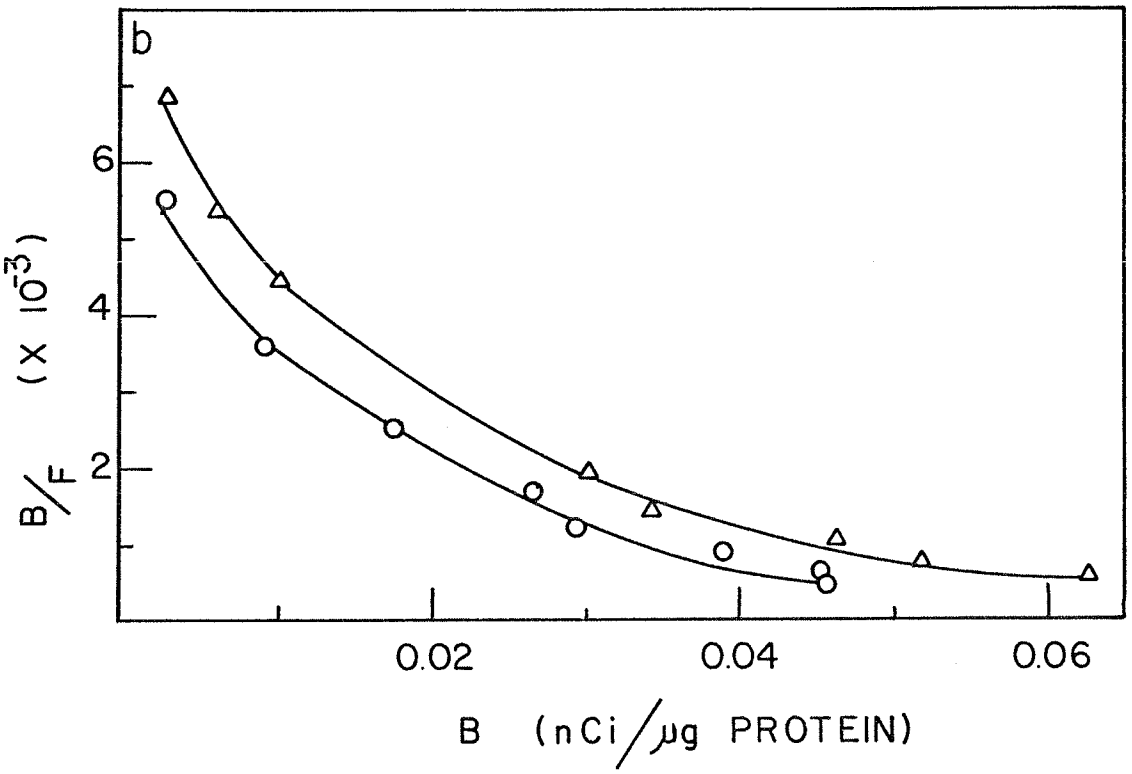
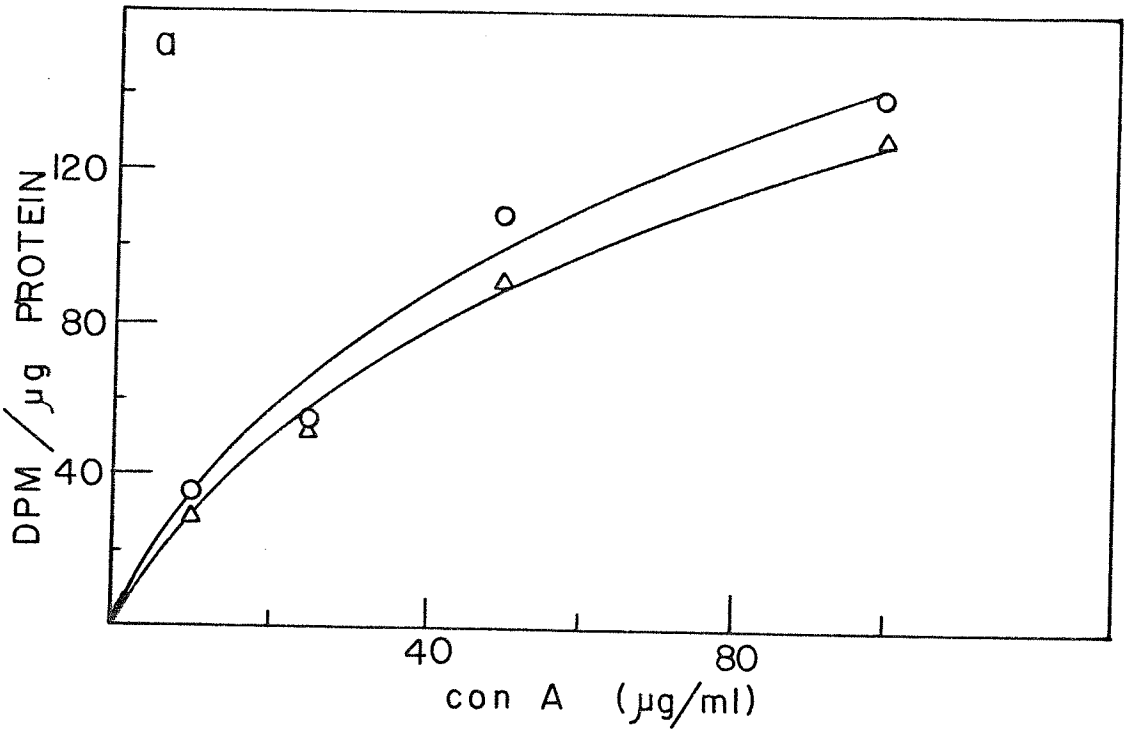

b
B/F (X 10⁻³)
6
4
2
0.02
0.04
0.06
B (nCi / µg PROTEIN)

Figure 50: Effect of various concentrations of [^{125}I] con A on binding to skin fibroblasts from a 12 year old Duchenne patient and an 11 year old normal child. o——o, GM 323, normal 11 year old male; Δ——Δ, Anderson, Duchenne dystrophic 12 year old male. (b) is a Scatchard analysis of the data presented in (a).


a
DPM / µg PROTEIN
120
80
40
40
80
con A (µg/ml)

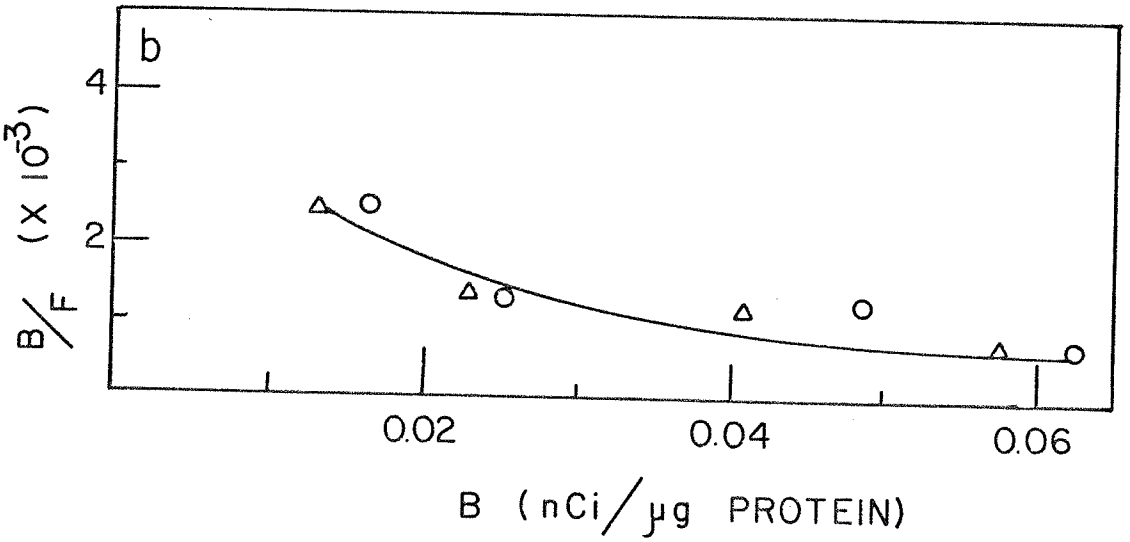
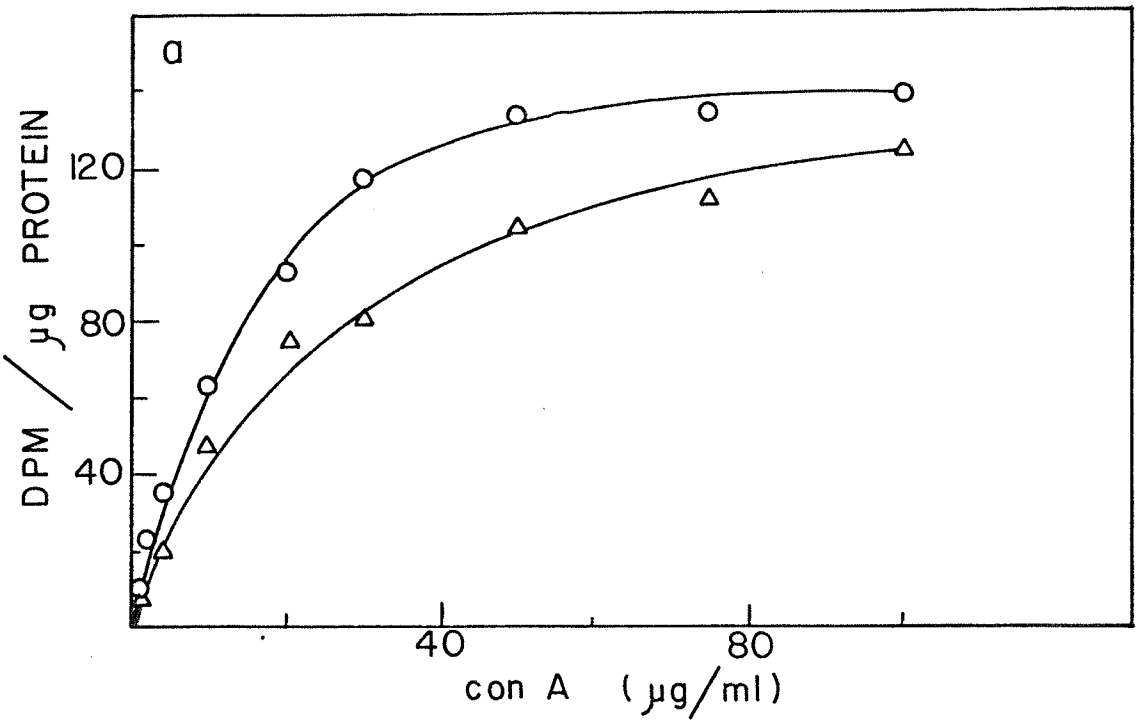
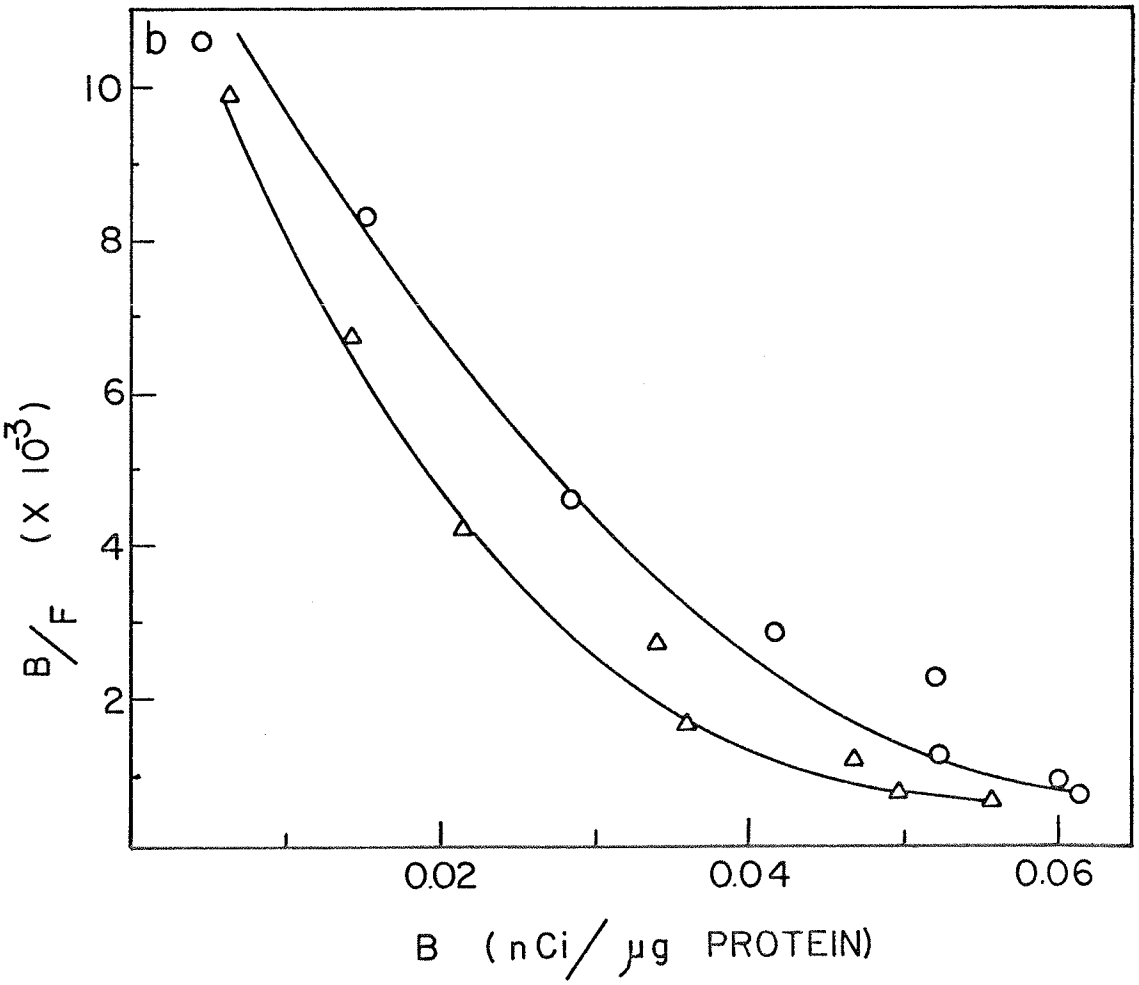

b
B/F (X 10⁻³)
4
2
0.02
0.04
0.06
B (nCi/µg PROTEIN)

Figure 51: Effect of various concentrations of [^{125}I] con A, on binding to skin fibroblasts from a 15.5 year old Duchenne patient and a 17 year old normal child. o——o, MCH 57, normal 17 year old male; Δ——Δ, WG 730, Duchenne dystrophic, 15.5 year old male. (b) is a Scatchard analysis of the data presented in (a).

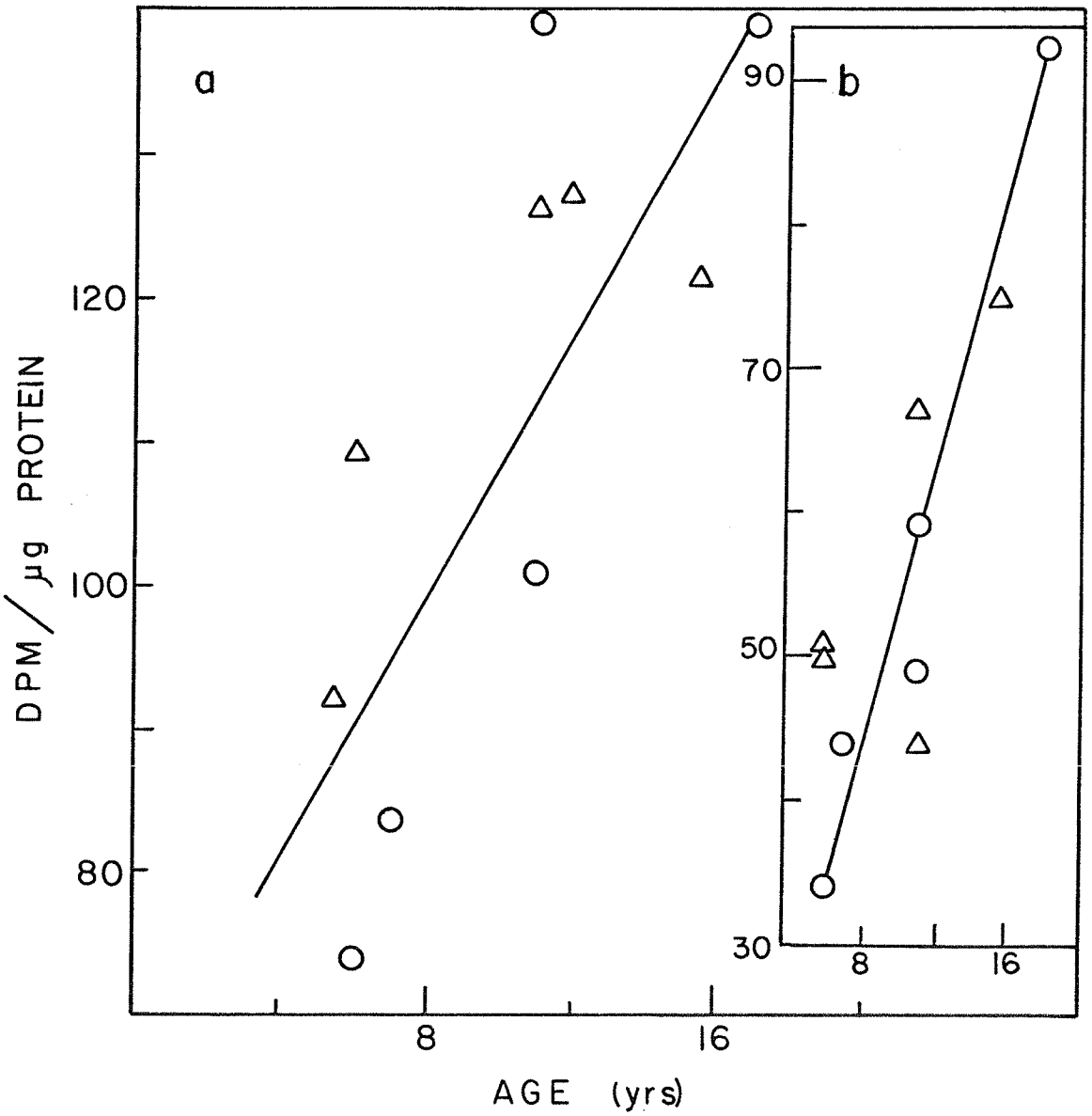

a
DPM / µg PROTEIN
120
80
40
40
80
con A (µg/ml)


b
B/F (X 10⁻³)
10
8
6
4
2
0.02
0.04
0.06
B (nCi/µg PROTEIN)

concentrations examined in Figures 47 to 51 did not reveal any striking differences in binding kinetics. Both normal and dystrophic fibroblasts contain con A receptors with similar affinities, although there apparently were some differences in total lectin receptors.

An increase in con A binding to fibroblast surfaces has been correlated with *in vitro* aging (increased passage number) (Aizawa and Kurimoto, 1979). In the present study [^{125}I] con A binding was also observed to increase with the age of the patient (Figure 52), thus confirming the idea of an age-dependent surface modification in skin fibroblasts. The increase in binding is two-fold over the age range represented by the fibroblast cultures studied, if measured at 20 µg/ml con A, and slightly less (1.5-fold) when measured at 100 µg/ml. No significant differences were detected for the Duchenne fibroblasts when compared to their normal control cultures.

Figure 52: Effect of increasing age on the binding of [^{125}I] con A to human skin fibroblasts. (a) 100 µg/ml con A; (b) 20 µg/ml con A. The data was taken from Figures 47 to 48. Δ, Duchenne muscular dystrophy patients; o, normal children.


a
b
120
100
80
90
70
50
30
8
16
8
16
DPM / µg PROTEIN
AGE (yrs)

DISCUSSION

DISCUSSION

An area of major interest in modern cell biology is directed toward uncovering the function of structures at the surface of the cytoplasmic membrane which control or participate in developmental processes. As outlined in the History section of this thesis, there is considerable evidence to implicate cell surface glycoproteins as being central components in many cellular interactions including differentiation. In particular, cellular adhesion and recognition appear to be specifically mediated by glycoproteins, and the oligosaccharide moieties are likely to be directly involved in providing cognitive information to specific protein structures.

Many genetically determined diseases, with as yet unknown causes, may be hypothesized to result from improperly functioning cellular interactions or from defects in developmental pathways. A valid approach to studying potentially important cellular structure: function relationships is to isolate somatic cells with mutations which affect these processes, in the hope that model systems may be developed *in vitro* to facilitate a more complete understanding of such diseases.

The L6 myogenic cell line (Yaffe, 1968) is frequently used for biochemical studies which seek to answer specific questions about the mechanism involved in the transition from a growing population of mononucleated single cells, to a multinucleated syncytium of terminally differentiated muscle tissue (see Pearson, 1980, for a review).

Genetic and functional homogeneity, high plating efficiencies and the immortal nature of this cell line all contribute to making it an attractive choice as a model system in which to genetically approach the study of myogenic differentiation.

Selection of Independent Con A-resistant Myoblasts

Mutants resistant to a variety of cytotoxic drugs have been selected and some interesting observations have been made on the nature of differentiation in the variant cells (see the section on myogenesis of permanent cell lines in the History section). However, many of these early studies suffer from the criticism that little was known about the rate at which non-fusing cells spontaneously appear in growing L6 myoblast populations. It is well known that passage of myogenic cultures at high density selects for cells unable to initiate differentiation (Yaffe and Saxel, 1977; Kaufman, 1978). At confluence, normal L6 myoblasts terminally suppress their proliferative capacity and induce a program of myogenic differentiation. Myoblasts which are changed in their requirements for myogenesis have a selective advantage for growth under these conditions, and rapidly accumulate while the normal cells proceed to differentiate. Upon subculturing a high density population, the non-myogenic cells make up a larger fraction of the viable inoculum. Therefore, the selection system designed to recover con A-resistant myoblasts in this study attempted to minimize the number of passages required between cloning a population of myoblasts, which were identified as possessing the ability to undergo myogenesis, and the identification of a con A-

resistant cell derived from that population. Considering that the populations of myoblasts used for mutant selections were grown from a single cell to a plate of 3 to 4 x 10^6 cells before selection, and that approximately one-half of a colony was allowed to differentiate before being grown to the larger population, it can be calculated that only 22 to 23 generations have elapsed before exposure to con A. Since the recovery frequency of the mutant in these populations was calculated to be 1 in 2.5 x 10^6 cells, (4 x 10^{-7}), the wild type population size reached before beginning selection represents the minimum size compatible with having a significant chance of recovering a mutant cell.

Many other workers have mutagenized, with methylating agents, their parental populations before beginning mutant selections (Loomis *et al.*, 1973; Merrill and Hauschka, 1978; Somers and Pearson, 1975; Crerar *et al.*, 1977; Pearson, 1980; Luzzati, 1974; Dufresne *et al.*, 1976). While this procedure can increase the number of mutants of a particular type, it adds to the number of cell doublings while treated cells recover and are grown to facilitate maximum expression of mutations that result from methylation of DNA bases. An added complication is the induction by the mutagen of second site mutations which might have deleterious effects on myogenesis. If the number of genes which control myogenesis is large, then mutagenesis at two sites becomes of concern. This possibility has been avoided in the selection of con A-resistant cells by avoiding any mutagenic treatment before selection. Examination of the wild type populations

from which mutants were selected indicates that the upper limit of cells having non-myogenic phenotypes is 1.3%. This agrees well with independent estimates of 3% (Loomis *et al.*, 1973) and 2% (Ng *et al.*, 1977).

Many mutant cell lines have been isolated from L6 wild type lines which retain the capacity to differentiate. For example, lines resistant to agents such as oubain (Ng, 1977; Luzzati, 1974) 8-azaguanine (Luzzati, 1974; Dufresne *et al.*, 1976; Rogers *et al.*, 1978), BUdR (Merrill and Hauschka, 1978) have been reported which suggests that selection protocols do not necessarily result in the recovery of cells defective in myogenesis. With care in the design of the selection scheme, the frequency of spontaneously arising non-myogenic cells can be kept low enough that it need not be a great concern in interpreting the effect of the genetic lesion on the myogenic phenotype.

Repeated selections from independent, wild type, myogenic lines can make the correlation between defective myogenesis and a genetic alteration very tight. With the probability of randomly selecting a spontaneous non-myogenic line reduced to approximately 1%, the possibility of selecting four such lines in a row becomes about 1 in 10^8 attempts. Four con A-resistant lines were selected in this study and all displayed a non-myogenic phenotype (Table I), suggesting that the genetic lesion directly responsible for the altered sensitivity to the lectin in some way also determines that the myoblast will not begin myogenesis upon reaching confluence in culture. If

one assumes that the five non-differentiating con A-resistant variants selected from wild type clone 6 (Figure 6) represent independent genetic events and not daughter cells of a mutation arising early in the growth of the wild type clone, then the possibility of all eight variants being due to the spontaneous appearance of non-fusing myoblasts becomes entirely insignificant at about 8 in 10^{16} selection attempts. At no time during this project were fusion elements ever observed in any of the resistant variants, which in some cases remained in culture for up to six months; therefore, it was clear that the non-myogenic phenotype was a stable property of these cell lines.

The variants that were selected by the procedure outlined in Figure 6 all display a modest 1.9- to 2.0-fold resistance to con A concentrations in the medium as measured by colony formation at the D_{50} level (Figure 7, Table I).

Low levels of resistance to cytotoxic agents make single-step selections somewhat more difficult than is the case for cells which display a high level of resistance. Cells displaying only a small resistance are susceptible to killing by the cytotoxic agent at concentrations which are necessary to reduce the plating efficiency of wild type cells to levels where variants cells become a significant fraction of the surviving population. If highly resistant cells are present in a population, it is possible to demonstrate a "triphasic" killing curve in response to increasing drug: 1) a plateau phase before cytotoxicity is produced, 2) an exponential decrease of

plating efficiency, and 3) a final plateau of relative plating efficiency due to colony formation by the highly resistant cells. This type of curve has been demonstrated in Chinese hamster ovary cells where resistance to WGA or PHA has been measured over a large range of concentrations (Stanley *et al.*, 1975a, Stanley, 1981). Selections may easily be performed in drug concentrations that produce the third phase of the survival curve. No myoblast colonies were routinely recovered when plates were incubated with 60 µg/ml in killing curves of wild type populations with a total of 2.5 x 10^6 cells. Thus, it seemed likely that either the mutants would be rare or would have relatively low resistance to the lectin.

Previous selections of con A-resistant Chinese hamster cells in this laboratory had indicated that a 2.5-fold change in resistance was the highest that might be expected (Ceri and Wright, 1978a) when D_{10} or D_{50} values for con A with variant and parental wild type were compared. These mutants occurred in mutagenized populations at a frequency suitable for recovery in a single step selection. Therefore, care was necessary in designing the selection protocol. It has since been shown in this laboratory that L6 myoblasts with very small relative resistances to the cytotoxic drug hydroxyurea can be selected by exposing wild type populations to a high drug concentration for only a short period of time. The variant may survive for longer times than the wild type cells under these conditions, but upon returning to low drug concentrations, begins growth more vigorously than the parental cells (Creasey, personal communication).

Examination of the killing curve presented in Figure 7 reveals that plating efficiency of the wild type myoblasts drops rapidly as con A concentrations increase above the D_{50} level. When the lectin concentration is doubled to 50 µg/ml, the D_{50} level of the resistant variant cells, plating efficiency of the wild type myoblasts is decreased to 10^{-5}. As shown in Figure 6 however, only an average of 8 out of 40 colonies which survive this concentration of con A give populations demonstrating efficient colony formation at twice the wild type D_{50} lectin concentration. 50 µg/ml con A was judged to be the optimum concentration for selection since it reduces the plating efficiency of the wild type to levels where relatively few are recovered, but at the same time allows efficient colony formation by the resistant myoblasts. Higher or lower concentrations of the lectin would result in killing most of the variants as well or, necessitate screening a greatly increased number of wild type survivors respectively.

Resistance to Con A Cytotoxicity

The results of binding radiolabelled con A to the cell surface of intact wild type and variant myoblasts clearly indicated that a relative resistance to the lectin was associated with a reduced binding of con A to the surface of the variant. When measured in the concentration range of 0.25 to 10 µg/ml con A, all four of the variant cell lines bound only approximately half the lectin bound by their parental wild type lines. This is in good agreement with the relative increase in resistance to the lectin in the growth medium,

which at a D_{50} concentration was also two-fold. Therefore, it is tempting to associate surface binding of the lectin with a role in the cytotoxic mechanism of con A. Even if the final target site were not a membrane component, the reduced interaction of lectin and membrane would be expected to concomitantly lower the intracellular accumulation of this macromolecule, allowing survival and growth of the myoblasts at higher external con A concentrations.

Reduced con A binding has been observed in other cell lines selected for resistance to this lectin. The Chinese hamster ovary cell lines selected in this laboratory (C^R7, BC^R2 and EC^R1) were also examined at 4^oC for their ability to bind radiolabelled con A (Wright and Ceri, 1977a,b). Wild type and con A-sensitive revertants bind con A in a positively cooperative manner, whereas the resistant variants bind con A non-cooperatively; in addition, C^R7 cells bind only 60% as much lectin per cell (or 40% as much lectin per unit surface area). This latter measurement is in keeping with a demonstrated 2.5-fold resistance in these cell lines (Ceri and Wright, 1978a). The cooperative kinetics observed in the hamster cell lines were not observed when the wild type or variant myoblast cells were observed under the same assay conditions. This difference in binding mechanism is probably due to significant dissimilarities in membrane structure and function between the two cell types. For example, measurement of [^{3}H] con A binding at 37^oC to the wild type and variant myoblast lines in the absence of the energy poison sodium azide did not reveal the expected increase of lectin association with the cells

(Figure 29). In contrast, measurement of con A binding to Chinese hamster ovary cells at physiological temperatures leads to a large increase in association of the lectin with cells over that observed at 4°C. At the higher temperatures the relative reduction of con A binding by variant cells, compared to their parental wild type lines, is masked (Wright, 1973; Noonan and Burger, 1973).

Pinocytotic uptake at room temperature or higher has been proposed as the major mechanism of cellular con A association in the Chinese hamster ovary cell line, and this form of internalization masks the differences in surface binding at 4°C (Storrie and Edelson, 1977). Apparently then, the L6 myoblast line is not active in pinocytotic uptake. If enhanced membrane fluidity is required for the pinocytotic mechanism in Chinese hamster ovary cells, the greater mobility of membrane glycoproteins may lead to receptor site redistribution in response to con A binding. The result is exposure of additional lectin binding sites on the hamster cells even when measured at 4°C. Therefore, direct comparison of the binding characteristics of myoblasts and Chinese hamster ovary cells is difficult because of the different mechanisms which might be involved.

The details of con A binding to other resistant variants has not been examined as thoroughly, but it is safe to generalize that reductions are observed. Con A-resistant lymphoma cell lines (Trowbridge *et al.*, 1978) bind approximately half of the con A bound to the surfaces of wild type cells when measured at the lectin concentration used in selecting these variants. The con A-resistant Chinese hamster ovary line selected by Briles (1978) has only been

examined at a single concentration, and the line was found to bind only 70% as much con A per mg of cell protein as wild type cells (Briles, 1982). The con A binding properties of the Chinese hamster ovary line resistant to con A described by Cifone and co-workers have not yet been described (Cifone *et al.*, 1976; 1979).

It is interesting to note that all of the con A-resistant mutants that have ever been isolated, in this or any other laboratory, have been relatively low in resistance to the lectin (2- to 3-fold). In comparison, mutant hamster cell lines resistant to ricin have been selected which withstand lectin concentrations as much as 500 times that required to kill the wild type lines (Gottlieb and Kornfeld, 1976). A possible explanation for this is that glycosylation defects which would greatly reduce con A binding to cell surface glycoproteins are incompatible with cellular viability.

An interesting comparison can be made between Chinese hamster ovary variants isolated in this laboratory and the myoblast variants, when binding at high lectin concentrations is considered. Assuming that con A has a tetrameric molecular weight of 108,000d, it was possible to calculate from extrapolation of the Scatchard plot (Figure 28) that near saturating lectin concentrations, wild type myoblasts bind 1.25×10^7 con A molecules per cell while con A-resistant myoblasts bind only 8.13×10^6 con A molecules per cell. This represents only a 33% decrease in binding compared with the 50% decrease measured in the range of 0.25 to 10 µg/ml con A. Con A-resistant Chinese hamster cells, however, display a two-fold reduction in binding sites at saturating concentrations when compared

with wild type cells (Wright and Ceri, 1977a,b). Thus lectin binding only correlates well with relative resistance at lower concentrations in the myoblast cell lines while the reverse is true in the hamster cell lines, pointing to further differences in the lectin binding mechanism in these two cell types.

The relative loss of receptor sites by the variant myoblasts seen at low concentrations of con A (Figure 26) but to a lesser extent at high concentrations of the lectin (Figure 27), was confirmed upon Scatchard analysis of the data. Wild type myoblasts give a set of points which can best be approximated as two intersecting straight lines. Since the slope of the line is the apparent association constant for lectin: receptor binding, the best explanation for the data from wild type cells is that there are two general types of con A-binding sites (high and low affinity sites) on the surfaces of these cells. Examination of the data from lectin-binding experiments with variant myoblasts revealed binding sites with only one major affinity constant. The calculated values from the slopes of the lines in Figure 28 are 1.12 μM^{-1} and 0.639 μM^{-1} for the high and low affinity sites of the wild type line and 0.637 μM^{-1} for the variant cells.

It is clear that the binding sites remaining associated with the surfaces of variant cells closely resemble the low affinity sites of the wild type cells. The suggestion presented by this data is that the relative increase in resistance shown by the variant cells was primarily due to an alteration high affinity surface receptors. Two

possible mechanisms could be hypothesized to account for this change: 1) a total loss of these receptor binding sites, or 2) conversion from sites of high affinity to ones of the low affinity type. To determine which of these two mechanisms was at work in the resistance phenomenon, it became necessary to investigate the glycoprotein composition of wild type and variant myoblasts (see the following section).

However, it was noticed during the lectin binding experiment that the type of serum used in the growth medium had a dramatic effect on the ability to demonstrate a relative decrease in the cell surface binding of [^{3}H] con A to variant cells. By culturing the myoblasts in medium supplemented with calf serum rather than foetal calf serum, the difference between wild type and variant lectin binding was extinguished (Figure 31). Approximately 12% and 60% more con A binding occurred on wild type and variant myoblasts respectively, when cultured in calf serum. It is likely that the cells incorporate a greater amount of serum glycoproteins into their membranes when cultured in calf serum and that due to a defect in glycoprotein biosynthesis, the variant myoblasts utilize a greater amount of the available serum glycoproteins. When cells were maintained in serum-free medium following growth in the presence of calf serum, differences in con A binding between variant and wild type cells returned. This can be interpreted to mean that there is a selective loss due to protein turnover, of calf serum glycoproteins on the myoblast cell surface, so that the differences in cell-synthesized glycoproteins are again revealed. The time which cells

were maintained in serum-free medium (16 to 18 hours) has been shown to be approximately twice the half life previously measured for fucosylated glycoproteins of L6 myoblast cell surfaces (Doetschman, 1980). This treatment would leave only one quarter of the serum derived proteins remaining, if all of their turnover rates are similar.

The composition of the medium did not extinguish the relative resistance of variant myoblasts to con A cytotoxicity when compared with the parental wild type lines. These surprising results suggest the existence of productive and non-productive binding sites at the cell surface, a phenomenon that has been observed in other studies (Gottlieb and Kornfeld, 1976). Further, the implication is that con A cytotoxicity is mediated by a specific set of lectin receptors and that the additional binding of con A to serum derived cell surface sites is not productive, in the sense that it does not mediate cytotoxicity. Therefore, the number of binding sites at the surface of con A-resistant myoblasts increases when grown in calf serum without a loss of relative lectin resistance.

While some other reports have mentioned effects of serum (Bornens *et al.*, 1976) or cholesterol (Marshall *et al.*, 1979) on the binding kinetics of con A to cell surfaces, this is the first observation which dissociates total cell surface binding of tetrameric con A from the cytotoxicity of this lectin. No detailed information is yet available as to the nature of the receptors involved or about their role in a mechanism in which con A kills cells.

Cell Surface Glycoprotein Alterations

The con A binding experiments predicted that variant myoblasts possessed altered surface oligosaccharides which interact with con A. This was confirmed by separation of glycoproteins by SDS slab gel electrophoresis after the oligosaccharide groups had been labelled by metabolic incorporation of [^{3}H] mannose from the culture medium. Repeated separations of purified surface membrane isolated in independent experiments gave a consistant pattern of Coomassie blue staining peptides that was not detectibly different between wild type and con A-resistant cell lines. Therefore, the differences which occur at the cell surface of the variant myoblasts must be of a minor nature when the overall protein composition is considered. This is in contrast to the Chinese hamster ovary cell lines resistant to con A isolated in this laboratory which express a major cell surface glycoprotein with a molecular weight of approximately 155,000d (Ceri and Wright, 1978b). This new protein accounts for as much as 15% of the total galactose oxidase-borotritiide label on $C^{R}7$ cells and is labelled by metabolic incorporation of [^{14}C] glucosamine only in these cells. It is likely that the appearance of such a major amount of new protein would be detectable by Coomassie blue staining as well. A major protein alteration has been detected in the Class E mutant mouse lymphoma cell lines possessing a pleiotropic glycosylation defect, which results in a reduced ability to bind con A (Trowbridge *et al.*, 1978a,b). The T25 (Thy-1) antigen is not expressed at the

cell surface of these variants and many other proteins appear to migrate slightly faster than their counterparts from parental cells. A similar faster migration rate could be seen for many cell surface proteins of the con A-resistant mutant CHO cells described by Cifone *et al.*, (1979). However, none of the Coomassie blue staining bands of the variant myoblasts isolated in this study appeared to be altered significantly in migration rate during electrophoresis.

The difference that was observed with the proteins separated by electrophoresis was the relative amount of [^{3}H] mannose incorporated into bands which migrate with molecular weights estimated to be 44,500d and 32,800d (Figures 35 and 36). The pattern of incorporation was complex and it is likely that many smaller changes would tend to be obscured. It must be remembered that although mannose generally enters cell surface glycoproteins through the formation of mannolipid intermediates or oligosaccharide-lipid complexes, a second pathway, by which label from [^{3}H] mannose can appear in glycoproteins without involving lipid intermediates is through conversion to GDP-[^{14}C] fucose (Rosen and Swartz, 1974). This adds further to the difficulty of interpreting changes in the pattern of radioactivity recovered from the gel. Wright *et al.*, (1979)have observed that label from GDP-[^{14}C]-mannose is converted to fucose and is incorporated into membrane bound glycoprotein in isolated membranes from wild type Chinese hamster ovary cells and this conversion is enhanced in variants selected for resistance to con A due to increased fucosyl transferase activity. The role

that is played in the relative resistance to the lectin by an increased content of fucose in membrane glycoproteins is unclear, but it is probable that increased fucosyl residue transfer to membrane glycoproteins affects the relative quantity or location of mannose. Since con A shows a high specificity for α-D-manno-pyranosyl residues, significant changes in lectin-cell membrane interactions might result. An analogous increase in labelled galactose utilization as glucose residues has been shown in the galactosylation-deficient Chinese hamster ovary cell line clone 13 (Briles, 1982). Here, UDP-galactose is converted by the action of a 4' epimerase to UDP-glucose, which in turn is utilized in the normal fashion in the variant cells, increasing the labelling of glucosylated products (Li *et al.*, 1978).

If the changes of mannose incorporation into lipid intermediates were to be important in the reduction of con A binding to variant myoblast membrane glycoproteins, then some differences in their interaction with the lectin should be demonstrable with isolated membrane proteins. "Staining" of the denatured proteins in the acrylamide gel with [^{125}I] con A revealed that all of the major binding proteins of the wild type remain as binding sites in the gel tracks of separated proteins from con A-resistant surface membranes (Figure 37). This was confirmed in two independent experiments; moreover, the total [^{125}I] con A bound to the gel track was similar for each cell line (Figure 38) - a direct contrast to the observations made on the binding at intact cell surfaces.

Therefore, the interaction of con A with the major band of [^{3}H]

mannose incorporation in the SDS-PAGE gels was examined in more detail. A pronase digest of the 44,500d region was found to contain a greater proportion of glycopeptides with lower affinity for con A sepharose columns than wild type preparations. The proportion of radiolabelled material which remained bound to the column after washing with buffer was similar in both wild type and variant cell lines. The situation is probably analogous to the binding of [^{125}I] con A to denatured glycoproteins within a polyacrylamide gel, where no differences are observed between wild type and variant. Of the label from variant myoblasts remaining bound to the con A sepharose column, the majority (95%) was released by 0.05 M αmethyl-D-mannoside. This is in sharp contrast to the pattern of elution of label extracted from wild type protein - only 30% being released at this point in the gradient. These results suggest that the proportion of high affinity lectin binding sites on con A-resistant myoblasts is significantly reduced. Importantly, these studies also support the binding data with whole cells, which indicated that variant cell surfaces appeared to have lost a set of high affinity sites found on parental wild type myoblasts.

The reduction of total binding sites which was observed by binding [^{3}H] con A to intact surface membranes was not reproduced in the binding studies with isolated protein. The membrane environment is undoubtedly more complex than the conditions which prevail within a polyacrylamide gel or a con A sepharose column. Interaction of the lectin with other protein or carbohydrate components within the intact membrane could lead to stearic hinderance which is reflected

as reduced binding to structurally altered oligosaccharides. This situation might be mimicked by including a hapten inhibitor in the buffer during con A binding to the acrylamide gel or during the initial absorption of mannose-containing glycopeptides to the con A sepharose column. Under these conditions, fewer of the low affinity oligosaccharides would be bound, reducing the total binding to a greater extent in glycopeptides from variant cells than wild type myoblasts. Figures 39 and 40 show that if this experiment were performed in the presence of 0.05 M α methyl mannoside, very few of the low affinity sites would successfully bind the labelled lectin.

An independent example exists of a lectin-resistant mutant cell line which exhibits a decreased ability to bind the lectin used in selection by virtue of a reduced affinity rather than a reduction in the numbers of binding sites. The WGA-resistant clone of mouse B16 melanoma cells binds about 60% of the lectin bound by parental line, but analysis of cell surface proteins labelled by the [^{125}I], lactoperoxidase method, then separated by WGA affinity chromatography and SDS-polyacrylamide gel electrophoresis, revealed that most of the WGA receptor glycoproteins could still interact with the lectin (Jumblatt *et al.*, 1980; Finne *et al.*, 1980). These results argued against major differences in the number of potential binding sites, but that the major difference was in the binding affinity of the cell surface glycoproteins which interact with WGA.

Biosynthesis of Lipid Intermediates of Protein Glycosylation

The cell surface glycoproteins with altered con A binding properties were determined to have arisen by impairment of the biosynthetic pathway leading to glycoprotein oligosaccharide structures. Membrane preparations from the four independently isolated con A-resistant myoblast lines incorporate significantly less GDP-[^{14}C] mannose into the lipid intermediates involved in protein glycosylation than preparations from parental wild type cells. Although it is possible that other factors such as levels of endogenous substrates or inhibitors and degradative enzymes in the assay system could influence results, it is likely that these effects would be small since glycosidase activity is low at pH 7.4 (Hellman *et al.*, 1982; Blaschuk *et al.*, 1980), and pyrophosphatase activity would be inhibited by UDP-N-acetyl-glucosamine and AMP in the assay (Vessey and Zakim, 1975). Since there are large differences in label incorporated from GDP-[^{14}C] mannose into fractions from wild type and variant cells, the simplest explanation for the results is that there is a defect in the formation of lipid intermediates in the con A-resistant cell lines.

One problem in all the experiments reported here is the inability to demonstrate incorporation of label into protein fractions in isolated membranes. Conditions under which mannosyl transferase activity is assayed are probably not sufficient to demonstrate the last step in the pathway *in vitro*. Thus assumptions about transfer to protein could not be examined directly in this system. The

transfer of mannose to glycoproteins could be detected in permeabilized whole cells, and in the initial studies, transfer appeared to be reduced in variant cells when compared to wild type cells (Table IX). Therefore, the final product is deficient in mannose residues, and this was confirmed in at least two membrane glycoproteins separated by SDS-polyacrylamide gel electrophoresis (Figures 35 and 36).

Similar observations have been made for the biosynthetic activities of other con A-resistant cell lines isolated by Wright and co-workers (Wright, 1979; Wright *et al.*, 1980) as well as lines isolated by other laboratories (Krag, 1979; Chapman *et al.*, 1980). The results of these studies have suggested that two independent biosynthetic defects may give rise to the con A-resistant phenotype. In Chinese hamster lines the lesion appears to be due to lack of free dolichol required for the transfer of the last five mannose residues to the lipid-oligosaccharide structure involved in protein glycosylation (Wright *et al.*, 1979; Krag, 1979). Therefore, con A-resistant membrane preparations incorporated approximately wild type levels of GDP-[^{14}C] mannose into lipid intermediates when exogenous dolichol-phosphate was supplied (Krag, 1979). However, in con A-resistant mouse lymphoma cells, exogenous dolichol-phosphate does not enhance mannose incorporation and the defect is assumed to result from an inactive mannosyl transferase enzyme (Chapman *et al.*, 1980). Addition of dolichol solubilized in dilute Triton X-100-containing buffer to preparations of membrane from wild type or variant myoblasts demonstrated that

mannosyl transferase activity could be stimulated up to 60-fold when lipids solubilized in $CHCl_3$:CH_3OH (2:1) were measured. The increase in activity is less than two-fold for the variant membranes (Table X). This experiment confirmed that the lesion resembles the putative transferase defect of the lymphoma variants.

One interesting observation that was made during the course of these studies was that the serum composition of the medium has a large effect on mannosyl transfer to oligosaccharide lipid fractions in isolated microsomal membranes. Calf serum has an inhibitory effect on this activity in both wild type and variant myoblasts (Figure 43), to the extent that no transfer could be detected in membranes from variant myoblasts cultured in calf serum, and wild type activity was reduced four-fold when compared with results obtained after growth in foetal calf serum. The nature of the regulatory stimulus in serum is unknown, but the possibility exists that it is related to the increased number of binding sites for con A that were shown to exist on surfaces of wild type and con A-resistant myoblasts after growth in calf serum (Figure 31). Regulatory changes in the synthesis of lipid linked oligosaccharides have been previously shown by Robbins *et al.*, (1977), who demonstrated the mannose content of the glucosylated oligosaccharides to be reduced when Chinese hamster ovary cells were incubated in glucose-deficient medium.

The changes in biosynthetic activity detected in the con A-resistant myoblasts are probably sufficient to explain the reduced interaction of con A with cell surface glycopeptides. Two mannosyl

donors are recognized as contributing to the synthesis of lipid-linked oligosaccharides. GDP-mannose and dolichol phosphoryl-mannose have both been shown to be donors in the synthesis of a range of oligosaccharides from 5 to 15 sugar residues in size (Chambers *et al.*, 1977; Forsee *et al.*, 1977; Kang *et al.*, 1978). Inhibition of the formation of dolichol phosphorylmannose with either EDTA or amphomycin reduced the maximum oligosaccharide size to seven sugar residues, suggesting that GDP-mannose could serve as a donor for lipid-linked oligosaccharides up to this size but that dolichol-P-mannose was required to form the larger species. In the con A-resistant myoblast lines, dolichol phosphoryl mannose synthesis appears to be blocked as a result of a genetic lesion in a mannosyl transferase activity. Consequently, the mannose containing oligosaccharides that continue to be synthesized and transferred to protein (Table IX; Figures 35 and 36) are likely to result from mannosyl transferase activities which directly utilize GDP-mannose as a donor. It is also likely that the size of the high mannose oligosaccharides would be reduced in the variant lipid-oligosaccharide and glycoprotein fractions. For example, the mouse lymphoma Thy 1^- mutant deficient in dolichol-P-mannose synthesis accumulates a lipid-linked oligosaccharide precursor which contains only five mannose residues instead of the normal nine and the smaller oligosaccharide is transferred to protein (Trowbridge and Hyman, 1979; Chapman *et al.*, 1979b). The 2-O-mannosyl linkages of the high mannose structures are one of the groups which determine a high affinity interaction with concanavalin A (Kornfeld and Ferris, 1975); however these are reduced in the five-mannose oligosaccharide

to only two linkages from a total of four in the normal nine mannose structure. In addition, the five mannose structure has only two non-reducing terminal mannose groups (the other mannose linkage which allows con A binding) while the wild type high mannose oligosaccharide may have three terminal mannose groups. Transfer of a similarly truncated oligosaccharide to the con A-resistant myoblast cell surface glycoproteins would be expected to reduce the affinity with which this lectin binds, as was demonstrated by the disappearance of high affinity sites from the surface of the variant cells, and by the increased ability of the hapten inhibitor α methylmannoside to reverse binding of isolated glycopeptides to con A sepharose columns. Nevertheless, sufficient binding to the smaller oligosaccharides would remain so that cytotoxicity of the lectin could be demonstrated at slightly higher lectin concentrations (approximately two-fold). Therefore the changes which bring about cellular resistance to the lectin result mainly from a reduced affinity for its receptors, as shown by the con A sepharose binding of isolated glycopeptides. To a lesser extent, a numerical reduction in the total lectin binding sites at the cell surface would also contribute to the resistance.

A Relationship Between Altered Glycoprotein Structures and Defective Myogenesis in L6 Myoblasts

The crucial question that must be answered is to what extent, if any, does the surface membrane regulate the myogenic program in

the L6 myoblast cell line? The results presented in this study have determined that there is a direct correlation between synthesis of altered cell surface glycoproteins that result in a con A-resistant phenotype, and an inability to undergo myogenesis in culture. In none of the eight con A-resistant myoblast cell lines which were isolated could membrane fusion or production of creatine kinase be measured. It would therefore appear that correct assembly of mannose-containing oligosaccharide structures is a prerequisite for expression of the developmental program.

Current models which describe the myogenic process in L6 myoblasts suggest that there is a mutual exclusion between production of the biochemical components specific to differentiated myotubes and continued proliferative capacity (Nadal-Ginard, 1978; Kaufman *et al.*, 1980). The seminal event which must occur, is withdrawal from the cell cycle into an extended G_1 phase, from which further changes in cellular activity "switch" the myoblasts to a terminally committed G_0 state, in which the differentiated functions such as fusion begin to be expressed. Some of the stages during cell membrane fusion have been defined by observing interactions between primary chick myoblasts (Knudson and Horwitz, 1977). The tentative sequence that has been outlined begins after the cells have committed themselves to differentiation with synthesis of specific cell surface components functioning in cellular recognition and subsequently, specific adhesion between the myoblasts. Only then can the components necessary for membrane destabilization and fusion interact to bring about formation of a syncytial cytoplasmic membrane. It is

likely that a similar sequence is operational in the L6 myoblast line.

L6 myoblasts however, only begin the differentiation process after reaching confluence on the culture substrate (Bignami *et al.*, 1982). Presumably a phenomenon related to contact inhibition of growth initiates the myogenic program. This is in contrast to primary myoblast lines which appear to follow a more programmed developmental sequence in culture. Under appropriate conditions, myogenesis proceeds after a suitable length of time whether or not cell divisions have been sufficient to produce a confluent monolayer (Konigsberg, 1979).

In the L6 myoblast line then, two points in the hypothesized sequence of events might be susceptible to perturbation, by alterations in the structure of surface membrane glycoproteins: 1) at the time which membrane contacts are established in a growing monolayer of cells and 2) during specific membrane recognition and adhesion events leading to membrane fusion after commitment to myogenesis.

The data collected in this study seem to reflect the former possibility. The two parameters which were routinely measured as indicators of muscle-specific differentiated functions were fusion of cytoplasmic membranes, and creatine kinase activity. These two muscle functions are closely regulated during differentiation *in vitro*, but are not necessarily coordinately controlled. For example, inhibition of membrane fusion in chick primary myoblasts does not prevent the appearance of this muscle protein (Burstein and Shainberg, 1979). Rogers *et al.*, (1978) have selected revertants of myoblast X fibroblast

non-fusing hybrids which were able to align and fuse, but are unable to make high levels of creatine kinase, indicating that regulation of the enzyme and fusion had become unlinked in this cell line.

In the con A-resistant myoblasts isolated during this study, both creatine kinase activity and membrane fusion are prevented from appearing after confluence. Creatine kinase is known not to be a glycoprotein (Watts, 1973) therefore it is certain that defective lipid-linked oligosaccharide biosynthesis would have no direct effect on this enzymatic activity. The more likely explanation would be that an event prior to production of differentiated functions has been disrupted, probably the initiation of events which promote withdrawal from the cell cycle and commitment to the developmental program.

The possibility that this early change which prevents withdrawal from the cell cycle in variant cells, is due to conversion to a transformed phenotype, was examined. A study of several cellular properties during culture *in vitro* which are indicative of the transformed phenotype revealed that the wild type and variant myoblasts are not substantially different. Plasminogen activator synthesis, growth in soft agar, and culture density at confluence all remain relatively unchanged in the con A-resistant myoblasts. Growth in medium supplemented with a low serum content (1%) also indicated that the growth regulation of the variant myoblasts was similar to the wild type cells (Figure 21). In both lines, exponential doubling times increased in response to the reduced serum,

and as saturation approached, a phase of very slow growth was initiated. Therefore, the ability of variant myoblasts to substantially increase the length of time spent in the G_1 phase of the cell cycle did not appear to be impaired. The variant myoblasts however, continued to grow very slowly beyond the time at which fusion had begun among wild type cells, presumably due to their inability to withdraw totally from the cell cycle and terminally initiate their developmental program.

It is interesting to note that few changes were detected in other growth characteristics of the con A-resistant myoblasts including growth rates, colony formation at elevated temperature, or collateral sensitivity to several membrane active agents. The latter two phenomena have been documented in Chinese hamster cells selected for resistance to con A (Wright, 1973; Ceri and Wright, 1978a), but may be due to a fundamentally different membrane structure upon which glycosylation defects have greater influence, or the nature of the lesion in oligosaccharide biosynthesis (Krag, 1979) alters glycoprotein function in a manner which is not encountered with the variant myoblasts. Limitation of the most dramatic phenotypic changes to the developmental competance of the con A-resistant myoblasts supports the argument favouring a major informational role of membrane glycoprotein oligosaccharide groups during myogenesis.

Suggestions for Future Experimentation on Cell Surface Glycoproteins Involved in Myogenesis

The selection scheme outlined in this thesis has led to a tight correlation between the con A-resistant phenotype and an inability to initiate myogenesis in the L6 myoblast cell line. Because of the care applied to both selection of independent wild type cell lines and the recovery of con A-resistant mutants from these parental cells, comparisons of many aspects of myogenesis or glycoprotein metabolism between wild type and variant cells will be valid tests of that correlation. An outline of some possible experiments which would extend the findings made in this study are suggested below.

Although the changes in glycopeptide affinity and cellular sensitivity to con A may be rationalized on the basis of the defect in the mannosyl transferase which was discovered in membrane preparations of variant myoblasts, an examination of the other enzyme activities of the glycoprotein biosynthesis pathway are required to fully test the idea that only a single enzymatic defect is responsible. If this is correct, then synthesis of oligosaccharide-lipid with radiolabelled precursors such as glucose or N-acetylglucosamine would not show large changes between wild type and variant. Simultaneous alterations in many enzyme activities would support alternative hypotheses which predict inhibition on the basis of: a change in the membrane environment; the presence of an inhibitory substance with a broad specificity for glycosyl transferase reactions; or possibly, coordinate regulatory control of these enzymes.

Some evidence is accumulating to support the idea that glycosylation defects alone can result in defective enzymatic activity or regulation (Hellman *et al.*, 1982, Blaschuk *et al.*, 1980). The con A-resistant myoblast cell lines secrete increased amounts of hexosaminidase activity into the culture medium when compared with their parental wild type lines. One explanation for the results is that there is a defect in the formation of the mannose-6-phosphate recognition signal (Sly and Fisher, 1982) for hexosaminidase which results in the enzyme being secreted from the cell. An acid α mannosidase activity is completely missing from the variant cells, but since there is no increase in external levels, the most likely explanation is a defective enzyme (Hellman *et al.*, 1982). The possible effects on glycoprotein structure resulting from these alterations have not yet been explored, but it is conceivable that they may be part of the con A-resistant, non-myogenic phenotype of the variant myoblasts.

Detailed structural studies on the oligosaccharide portions of the glycopeptides and lipid-linked intermediates of variant and wild type cells will be required to determine the exact nature of the oligosaccharide alterations which have been detected in this study. It will be interesting to compare these structures with the truncated oligosaccharides synthesized by the lymphoma cell mutants isolated and studied by Trowbridge and his co-workers (Chapman *et al.*, 1979a,b). One difficulty that must be overcome before such a biochemical analysis could become feasible is the preparation of large

quantities of radiolabelled oligosaccharides from the myoblasts. Preliminary experiments in which [^{3}H] mannose was incubated with whole cells for periods under one hour gave preparations of lipid intermediates containing little radiolabel. Longer incubations complicate interpretation of results due to metabolic conversion of mannose to other sugars. Therefore, the encouraging results obtained with whole, permeabilized myoblasts in the synthesis of both lipid intermediates and protein products (Table IX) could be examined further as a source of labelled material. Some work would be necessary to optimize conditions for utilization of radiolabelled GDP-mannose as the substrate in these reactions, but the relatively large amount of product obtained with short incubation times could prove to be an excellent way in which to both prepare the biosynthetic intermediates, and to investigate the enzymatic activities of the pathway.

More knowledge will also be required about the types of mannosylated membrane glycoproteins which are involved directly in myogenesis. Very recently, Senechal *et al.*, (1982) observed that many cell surface glycoproteins which bind con A are specifically regulated during myogenesis. A similar study with the wild type and con A-resistant myoblasts isolated in this laboratory could help to define glycoproteins that are likely to be components of the myogenic process. For example, are there differences between the wild type and variant myoblasts in the appearance or turnover of any con A binding proteins as the cells reach confluence, or after that time - as wild type cells begin to fuse and the variant myoblasts remain as a confluent monolayer?

Other experiments will be required to more firmly establish a causative link between defective oligosaccharide-lipid biosynthesis and altered myogenic development in con A-resistant myoblast lines. The strict correlation shown in the experiments reported in this thesis suggest that a causative relation does exist. Reversion to a con A-sensitive state and concomitant recovery of myogenic capability by the resistant myoblasts would strengthen even further the link between glycoprotein structures at the cell surface and the process of myogenesis. No differences in the sensitivity of wild type or variant cells to membrane active agents, or other conditions large enough to be the basis for a revertant selection scheme were found during this study. Increased fucose content in membrane glycoproteins may make the variant cells more sensitive to cytotoxic lectins of this sugar specificity. The lectin from *Ulex europeus* seeds was tested, but was not cytotoxic until a concentration of 200 µg/ml was reached, suggesting that specific fucose binding may not have been involved in the killing effect. No difference in sensitivity was observed between wild type and variant myoblasts. It would be interesting to examine the relative sensitivity of these myoblast lines to the fucose binding lectin from *Lotus tetragonolobus*, a lectin which has been used for selecting and characterizing variants in other studies (Briles, 1982).

Phenotypic reversion of the defective myogenic phenotype and cellular resistance to con A may be attempted by supplying the variant myoblasts with the missing lipid-linked oligosaccharide

intermediates which were shown in this study to be incorrectly synthesized. If such an experiment were successful, it would be strong evidence for a causative link with myogenesis. This type of experiment has been performed with developing sea urchin embryos treated with compactin to inhibit gastrulation. Compactin arrested dolichol biosynthesis in the embryos (Schwarz and Datema, 1980), but addition of exogenous dolichol to the medium allowed gastrulation to proceed normally in the presence of the inhibitor (Heifetz and Lennarz, 1979).

Application of somatic cell genetics methodology to myogenic development is only in its beginning stages. Many questions remain open to investigation by this approach. The experimentation presented in this thesis has implicated the involvement of cell surface glycoprotein as an important regulatory component in initiation of the developmental program. This idea is based upon surface alterations which have been selected by a single lectin, concanavalin A. A large amount of information is beginning to collect regarding the interesting variety of structural alterations in oligosaccharide chains which can be selected in culture by employing other cytotoxic lectins (Wright, 1979; Wright *et al.*, 1980; Briles, 1982). What effects would other oligosaccharide truncations and modifications have on myogenic development in the L6 and L8 cell lines? An attempt could be made to answer this question by selecting a large panel of lectin-resistant myoblasts. It is already suspected that not all lesions in glycoprotein biosynthesis will have as severe an affect as has been shown for the

mannosyl transferase defect selected in this work. Some WGA-resistant mutants have been isolated which retain their ability to fuse in culture (Gilfix and Sanwal, personal communication).

A careful examination of the correlation between resistant phenotypes selected by many kinds of lectins or other membrane-active toxins, by the methodology outlined in this thesis, could lead to a much clearer understanding of the range of cell surface components which play a role during myogenic development in the L6 myoblast line.

Studies on [^{125}I] Con A Binding to Skin Fibroblasts From Normal Children and From Patients With Duchenne Muscular Dystrophy

When compared to normal skin fibroblasts under identical culture conditions, the fibroblasts from patients with Duchenne muscular dystrophy did not exhibit altered con A binding kinetics. Differences in the amount of lectin bound to the cell surfaces at 4^{o}C could be observed, but were not consistent among the five age-matched normal and diseased pairs which were examined. This variation suggests that individual fibroblast populations from separate sources have slightly differing lectin binding abilities when considered on the basis of the amount of lectin bound/μg of cellular protein.

The binding kinetics near saturation appeared similar to the results previously reported by Feller *et al.*, (1979) for con A, using nearly identical binding assay conditions with normal human skin

fibroblasts. However, when a comparison of con A binding at two concentrations was made among all the cultures examined in the experiments reported here, a distinct increase in the amount of lectin bound was observed as the age of the donor increased. A similar observation has previously been made using a con A-mediated red blood cell rosette formation assay of fibroblast surfaces (Aizawa and Kurimoto, 1979). One theory which has been advanced to explain the pathogenesis of Duchenne dystrophy is that this disease is a specific progeroid syndrome. Some observations on increased cell size, longer doubling times, and elevated 5'-nucleotidase activity in these cells tends to support the idea of prematurely aging cells (Liechti-Gallati *et al.*, 1981). Other workers could not support this hypothesis based on observations of culture life span and growth kinetics with normal and dystrophic fibroblasts(Wertz *et al.*, 1981). The results of con A binding to attached fibroblasts obtained in this study at $4^{o}C$ also do not support the premature aging hypothesis of muscular dystrophy.

The amount of $[^{125}I]$ con A binding to the cell surfaces of the skin fibroblasts was routinely calculated on the basis of total protein per 60 mm dish at cell monolayer confluence. When con A binding per dish was examined however, an interesting observation was made. The cultures of Duchenne fibroblasts always bound more lectin than the control cultures (a 6 to 55% increase, with an average of 25% more in six experiments). No attempt was made to determine the number of cells per plate in these experiments. Cell number does have an

effect on the total amount of lectin bound per plate (Figure 46), and must be considered as a possible reason for the consistently observed increase in lectin binding. Liechti-Gallati *et al.*, (1981) have suggested that the average cell size of Duchenne fibroblasts may be larger than normal cells. The increase in con A binding to dystrophic cells may be a reflection of that difference. This point bears further investigation by correlating total con A binding to cell number and membrane surface area.

Concluding Comments on the Molecular Genetics Approach to Glycoprotein Involvement in Myogenesis

The fundamental mechanisms involved in cell cycle withdrawal, initiation of differentiation and production of the final differentiated state by myoblasts are amenable to investigation by selecting and studying specific mutations which alter these cellular programs. Genetic manipulation of specific surface components provides an approach to investigating the involvement of surface molecules in the process of myogenesis. Previous studies in this laboratory have shown that the alterations in glycoprotein biosynthesis, which accompany con A resistance markedly change many biological properties of the cell (Wright, 1979; Wright *et al.*, 1980). These studies suggested that a similar lesion due to con A resistance in myoblasts would result in changes at the cell surface that could have profound effects upon the differentiation of myoblasts.

The results presented in this thesis and reported elsewhere (Wright *et al.*, 1980; Parfett *et al.*, 1981) support this initial hypo-

thesis and provide a link between con A-resistance, cell surface glycoprotein alterations, defective mannosyl transferase activity in myoblasts, and the inability to undergo normal cellular differentiation to form multinucleated myotubes. These observations emphasize the importance of cell surface glycoproteins in the process of myogenesis, and suggest that mannose may play a special role in this process.

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